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Morphogenesis of Mammalian Endoplasmic Reticulum and Golgi Apparatus throughout the Cell Cycle

MAIJA PUHKA

Academic dissertation

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Cover image: Puromycin treated CHO-K1 cells expressing endoplasmic reticulum markers were subjected to confocal microscopy (upper left), transmission electron microscopy (upper right) and electron tomography.

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It took a long time,
I moved three times, jammed my neck twice,
broke an Achilles tendon, broke up,
got married, two cats, one and a half child,
I smashed, cleared and passed
(floor ball, badminton, lab bench, you name it!)
I was frustrated, furious,
enjoyed and inspired,
but seldom bored
After all, I have learned,
not all good things come fast.
M.P.

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I

Puhka M, Vihinen H, Joensuu M, Jokitalo E. Endoplasmic reticulum remains continuous and undergoes sheet-to-tubule transformation during cell division in mammalian cells.

J. Cell Biol. 2007 Dec 3;179(5):895-909.

II

Puhka M, Vihinen H, Joensuu M, Belevich I, Novak I, Jokitalo E. Proportion of intact or fenestrated sheets and tubular ER varies between mammalian cell types and cell cycle stages and correlates with ribosomal density. Manuscript.

III

Uchiyama K*, Totsukawa G*, **Puhka M***, Kaneko Y, Jokitalo E, Dreveny I, Beuron F, Zhang X, Freemont P, Kondo H. p37 is a p97 adaptor required for Golgi and ER biogenesis in interphase and at the end of mitosis. Dev. Cell 2006 Dec;11(6):803-16.

***Equal contribution**

The thesis contains additional unpublished data.

Abbreviations and acronyms

BSA	**B**ovine **s**erum **a**lbumin
Cdc-2	**C**ell **d**ivision **c**ontrol protein **2** homolog
CHO-K1	**C**hinese **h**amster **o**vary **K1**
CLEM	**C**orrelative **l**ight **e**lectron **m**icroscopy
CLIMP-63	**C**ytoskeleton-**li**nking **m**embrane **p**rotein of **63** kDa
COP	**Co**at **p**rotein
COS-7	**C**V-1 cell line in **O**rigin carrying the **S**V40 DNA, **7**
GFP	**G**reen **f**luorescent **p**rotein
GM130	**G**olgi **m**atrix protein of **130** kDa
GRASP	**G**olgi **rea**ssembly **s**tacking **p**rotein
EM	**E**lectron **m**icroscopy
ER	**E**ndoplasmic **r**eticulum
ET	**E**lectron **t**omography
HeLa	Cervical cancer of **He**nrietta **La**chs
Huh-7	**Hu**man **h**epatocellular carcinoma **7**
HPF/FS	**H**igh **p**ressure **f**reezing and **f**reeze **s**ubstitution
HRP	**H**orse **r**adish **p**eroxidase
IC	**I**ntermediate **c**ompartment
INM	**I**nner **n**uclear **m**embrane
KASH	**K**larsicht, **A**NC-1, **S**yne **H**omology
LBR	**L**amin **b**eta **r**eceptor
LM	**L**ight **m**icroscopy
NE	**N**uclear **e**nvelope
NEBD	**N**uclear **e**nvelope **b**reak**d**own
NRK-52E	**N**ormal **r**at **k**idney **52E**
NSF	**N**-ethyl maleimide **s**ensitive **f**actor
Nup	**Nu**cleoporin **p**rotein
ONM	**O**uter **n**uclear **m**embrane

p97	Valosin-containing **p**rotein of **97** kDa
PBS	**P**hosphate **b**uffered **s**aline
PDI	**P**rotein **d**isulphide **i**somerase
PFA	**P**ara**f**orm**a**ldehyde
REEP	**R**eceptor **e**xpression **e**nhancing **p**rotein
RER	**R**ough **e**ndoplasmic **r**eticulum
SEM	**S**canning **e**lectron **m**icroscope
SER	**S**mooth **e**ndoplasmic **r**eticulum
ss	**s**ignal **s**equence
STIM-1	**St**romal **i**nteraction **m**olecule **1**
ST-HRP	α2,6-**s**ialyl**t**ransferase coupled to HRP
SUN	**S**ad1p/**UN**C-84
TEM	**T**ransmission **e**lectron **m**icroscope
TGN	***T****rans*-**G**olgi **n**etwork
TRPP-2	**T**estosterone-**r**epressed **p**rostate **p**rotein-**2**
VAP-B	Vesicle associated membrane protein (**V**AMP) **a**ssociated **p**rotein **B**
VCP	**V**alosin-**c**ontaining **p**rotein 97
VCIP135	**V**CP[p97]/p47 **c**omplex-**i**nteracting **p**rotein of **135** kDa
Vero	Kidney cells of African green monkey, **Ver**da Ren**o** (esperanto)
wt	**w**ild **t**ype

Summary

The endoplasmic reticulum (ER) and the Golgi apparatus are organelles that produce, modify and transport proteins and lipids and regulate Ca^{2+} environment within cells. Structurally they are composed of sheets and tubules. Sheets may take various forms: intact, fenestrated, single or stacked. The ER, including the nuclear envelope, is a single continuous network, while the Golgi shows only some level of connectivity. It is often unclear, how different morphologies correspond to particular functions. Previous studies indicate that the structures of the ER and Golgi are dynamic and regulated by fusion and fission events, cytoskeleton, rate of protein synthesis and secretion, and specific structural proteins. For example, many structural proteins shaping tubular ER have been identified, but sheet formation is much more unclear. In this study, we used light and electron microscopy to study morphological changes of the ER and Golgi in mammalian cells. The proportion, type, location and dynamics of ER sheets and tubules were found to vary in a cell type or cell cycle stage dependent manner. During interphase, ER and Golgi structures were demonstrated to be regulated by p37, a cofactor of the fusion factor p97, and microtubules, which also affected the localization of the organelles. Like previously shown for the Golgi, the ER displayed a tendency for fenestration and tubulation during mitosis. However, this shape change did not result in ER fragmentation as happens to Golgi, but a continuous network was retained. The activity of p97/p37 was found to be important for the reassembly of both organelles after mitosis. In EM images, ER sheet membranes appear rough, since they contain attached ribosomes, whereas tubular membranes appear smooth. Our studies revealed that structural changes of the ER towards fenestrated and tubular direction correlate with loss of ER-bound ribosomes and vice versa. High and low curvature ER membranes have a low and high density of ribosomes, respectively. To conclude, both ER and Golgi architecture depend on fusion activity of p97/p37. ER morphogenesis, particularly of the sheet shape, is intimately linked to the density of membrane bound ribosomes.

Introduction

1. Introduction to endoplasmic reticulum and Golgi morphogenesis

The mammalian cell is composed of proteins, lipids, carbohydrates and nucleic acids in water. These constituents are needed for all cellular functions including survival, multiplication, apoptosis, mobility, communication and the specialized tasks of different cell types. They are produced and delivered to various destinations in and on membranous organelles or vesicles (Palade, 1975; Farquhar and Palade, 1981), which allows separation of different tasks into efficient units (Fig. 1). Organelles are variable in shape, size and origin, each structure serving their specialized task, but like other cellular components, are constantly renewed and often reorganized. The basic membrane shapes found in organelles are flat sheets, fenestrated sheets, tubules, branch points and spheres (Fig. 1).

1.1 Basics of endoplasmic reticulum and Golgi structure and function

The endoplasmic reticulum (ER) is an organelle that synthesizes and modifies lipids, membrane and secretory proteins as well as proteins destined to the lumen of various organelles. In addition to these functions, it controls the quality of proteins and cytoplasmic Ca^{2+} levels, metabolizes drugs and toxins, contributes to regulation of gene expression and possibly to transduction of signals from extracellular matrix to the nucleus. Thus, it is the largest, most multidisciplinary and wide spread organelle within the cell. Because of its many fundamental functions, ER dysfunction is the reason for numerous diseases including particularly neurodegenerative, muscle and chronic metabolic diseases (Starr and Fridolfson, 2010; Hummasti and Hotamisligil, 2010).

Structurally, the ER is a complex network composed of sheets - that may be single, stacked, intact or fenestrated- and tubules that are connected to each other at branch points and to the nuclear envelope (NE) (Fig. 1 and 2A; Palade, 1956). The spherical vesicles made with the help of coat protein II, or more tubular carriers, are formed in a specialized subdomain

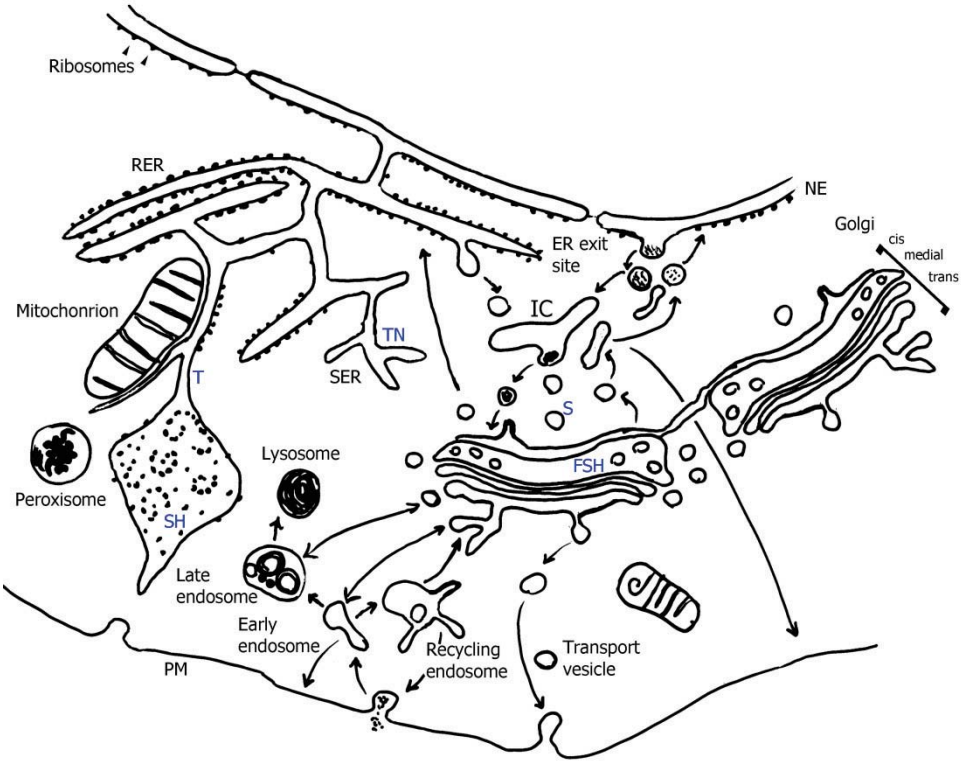

Figure 1. Organelle shapes and transport routes within cells. The basic shapes observed within the cytoplasmic organelles are sheets (SH), fenestrated sheets (FSH), tubular networks (TN), tubules (T) and spheres (S). An example is given for each of these shapes in the image. (B) ER bound ribosomes synthesize proteins, which are packed along with other cargo to carriers that bud from the ER exit sites. The cargo continues via intermediate compartment (IC) to Golgi or plasma membrane (PM) and from Golgi to PM or endo/lysosomal system. Retrograde routes are also indicated. RER, rough endoplasmic reticulum, SER, smooth endoplasmic reticulum. Drawn by the author according to Bonifacino and Glick (2004) and Marie *et al.* (2009).

of ER, ER exit site (Mironov *et al.*, 2003; Zeuschner *et al.*, 2006; Palmer and Stephens, 2004). From there, the carriers are targeted down the secretory pathway (Fig. 1) to the intermediate compartment (IC) and the Golgi apparatus (Saraste and Kuismanen, 1984; 1992; Marie *et al.*, 2009). The carriers contain proteins and lipids, which are further modified, sorted, packed and targeted to different destinations within the cell by the Golgi. Misplaced cargo can be returned to ER from either IC or Golgi.

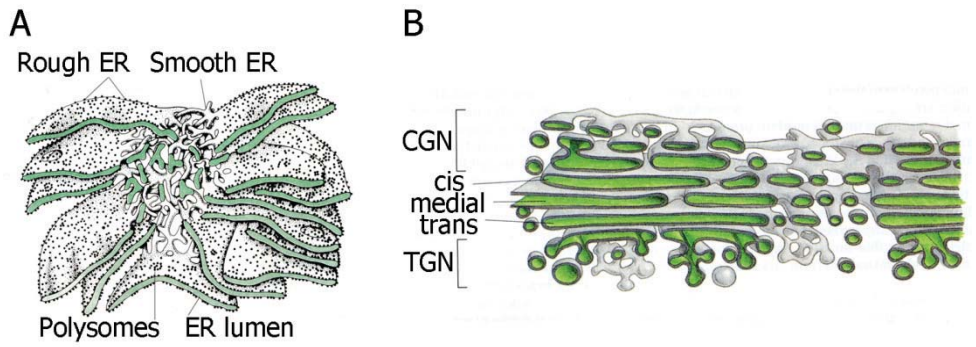

Figure 2. ER and Golgi structures in 3D. Both images are derived and modified from Molecular Biology of the Cell (Alberts *et al*., 2008), where ER image is after Rambourg and Clermont (1990) and Golgi image after Krstic, Ultrastructure of the Mammalian Cell (1979). *Cis*-, *medial*- and *trans*-Golgi cisternae are indicated. CGN, *cis*-Golgi network, TGN, *trans*-Golgi network.

Similarly to the ER, the Golgi is an interconnected membrane system that is made of sheets, more commonly known as cisternae –that are usually stacked and fenestrated- and tubules that form connections between equivalent cisternae in adjacent cisternal stacks. (Fig. 1 and 2B; Farquhar and Palade, 1981; Rambourg *et al.*, 1987; Sesso *et al.*, 1994; Ladinsky *et al.*, 1999). Tubular networks surrounding the stack on each side and tubular connections between different cisternae have been documented, although their existence or prevalence is a matter of some controversy (Rambourg *et al.*, 1987; Griffiths *et al*., 1989; 1995; Ladinsky *et al*., 1999; Emr *et al*., 2009). Vesicles, for example coat protein I (COPI) or clathrin coated, containing cargo or Golgi resident proteins shed from the edges of Golgi cisternae as well as tubular parts (Ladinsky *et al.*, 1994; 1999; Staehelin and Kang, 2008; Emr *et al*., 2009). However, unlike the ER, the Golgi is organized as a ribbon for which a wide variety of morphological determinants, such as Golgi matrix proteins, have been found (Wei and Seemann, 2010). It reserves a relatively small area residing usually on the pericentriolar side of the nucleus, and its integrity and positioning appear to be important for cell polarization.

The tasks of the Golgi are clearly organized into different subdomains with defined boundaries (Dunphy and Rothman, 1985). The Golgi contains a specialized set of enzymes that locate to different cisternae and process the target molecules in a sequential manner, when cargo proceeds from *cis*- to *trans*-Golgi. The current view favors cisternal maturation

model, where the same cisterna carries and processes the cargo with a changing set of enzymes all the way through the Golgi (Emr et al., 2009). For example, the Golgi affects structure of the target molecules by adding, removing or modifying the basic glycosyl groups added in the ER and by phosphorylation and proteolytic processing. Some modifications relate to the final function of the molecule, but others are made to target the molecule to a specific site within the cell. The *trans*-Golgi network (TGN) is the main domain, where modified cargo is sorted and packed into vesicular carriers for delivery to the target destination (Fig. 1). However, it has recently been shown that some of the cargo that leaves ER avoids entering Golgi altogether and is delivered from IC to plasma membrane directly or via endosomal system (Sannerud *et al*., 2006; Marie *et al*., 2009).

1.2 Energetics of shape formation

The majority of the lipid bilayers from which the organelle membranes are made of, are flat or of low curvature in nature (Lingwood *et al.*, 2009). This is surprising regarding the fact that most biological lipids form high curvature structures in lipid-water systems *in vitro*. However, it has been estimated according to the elastic model of lipid membranes (Helfrich, 1973) that bending of a lipid bilayer requires energy in correlation with the induced membrane curvature: tubule formation uses less energy than vesicle formation (Shibata *et al.*, 2009). Similarly, fusion and fission of membranes requires energy. The fusion reaction of ER derived microsomes and subsequent formation of sheets (Lavoie *et al.*, 1996, Dreier and Rapoport, 2000) as well as NE formation from microsomes (Hetzer *et al.*, 2001; Anderson and Hetzer, 2007) is powered by GTP *in vitro*. ER and Golgi tubule formation, on the other hand, spends energy in the form of ATP (Lavoie *et al.*, 1996; Dreier and Rapoport, 2000; Banta *et al.*, 1995). However, *in vivo* data is not quite consistent with these results. ATP depletion for up to 30 min in COS-7 cells did not result in gross alteration of the tubular ER morphology (Shibata *et al.*, 2008), whereas a 2 h depletion in Hela cells caused a large-scale tubulation of the ER (Lingwood *et al.*, 2009).

2. Proteins in membrane shape formation, spreading and dynamics

There are at least four general mechanisms to shape membranes (Zimmerberg and Kozlov, 2006; Shibata *et al.*, 2009; Graham and Kozlov, 2010): 1) scaffolding, 2) fusion and fission or 3) generation of asymmetrical lipid monolayers and 4) application of forces to pull membranes. The varying shapes and quantity of lipids in different lipid monolayers may contribute to generation of asymmetrical monolayers (Zimmerberg and Kozlov, 2006; Graham and Kozlov, 2010). The Golgi and ER have quite different lipid profiles, since there is an increasing gradient of sterols and spingolipids along the secretory pathway: starting from the ER, proceeding via the Golgi to the plasma membrane (van Meer and Vaz, 2005). The ER, being poor in cholesterol and rich in glycerolipids, might therefore have different membrane dimensions than the Golgi, which synthesizes sphingolipids. However, the prevailing view is that although lipids affect protein function and proteins move lipids between membrane monolayers, membrane shape generation is largely dependent on proteins consuming energy for the task (Zimmerberg and Kozlov, 2006; Graham and Kozlov, 2010).

2.1 Sheet formation

There is considerable variability regarding the amount of fenestrations, size and stacking characteristics of ER and Golgi cisternae within different cells. Despite many descriptions of structural details, in many cases it is unclear what purpose the structural variations serve.

2.1.1 Peripheral endoplasmic reticulum sheets

Peripheral ER sheets have long been described as rough, ribosome containing flat membranes. However, they may contain specialized smooth subdomains such as in ER exit sites (Bannykh *et al*. 1996), close contact sites between ER and Golgi (Ladinsky *et al*., 1999) or Ca^{2+} intake sites at the plasma membrane (Orci *et al.*, 2009). While factors contributing to the specialized smooth domains are quite well known, the structural determinants of the basic ribosome containing flat sheet morphology have only begun to emerge.

p180 is an ER protein that was found to bind ribosomes and microtubules and promote secretion and proliferation of the ER in monocytic leukemia cells (Ogawa-Goto *et al*., 2007; Benyamini *et al.* 2009) and, artificially, in yeast (Becker *et al.*, 1999). In mammals, p180 has many splice variants and its expression levels range from high in actively secreting tissues to negligible in several cell types (Langley *et al.*, 1998). The ability of p180 to bind ribosomes and induce RER proliferation seems to vary according to the splicing pattern that dictates the number of ribosome binding repeat domains (Bai *et al.*, 2008).

p180 is normally not expressed in yeast (Becker *et al.*, 1999). However, ectopic expression of different domains in yeast gave an idea, how p180 may be involved in formation of sheet structures. The N-terminus containing the transmembrane domain was found to be responsible for membrane proliferation. Interestingly, the proliferating membrane type was ruled by the ribosome binding domain: overexpression of constructs containing it resulted in amplification of RER, whereas constructs lacking it induced smooth membranes, also called karmellae (Wanker *et al.*, 1995; Becker *et al.*, 1999).

Induction of p180 in mammalian cells by overexpression led to proliferation of RER (Benyamini *et al.*, 2009) or conversion of existing ER membranes to RER when p180 was upregulated by ascorbate stimulation (Ueno *et al.*, 2010a). The ER appeared as long cross-sectional profiles in EM images (Benyamini *et al.*, 2009). In contrast, knockdown of p180 produced ER membranes that were organized as long lines of short profiles in EM images, which was interpreted as vesiculation, and the profiles contained a reduced number of ribosomes (Benyamini *et al.*, 2009). Moreover, the ER retracted towards the nucleus (Ogawa-Goto *et al.*, 2007). A similar effect was observed in structure of the Golgi apparatus: the usual long Golgi cisternae transformed into a collection of short, although still stacked membrane profiles upon p180 depletion (Benyamini *et al.*, 2009). Ueno and collegues (2010b), on the other hand, noticed a preferential effect on TGN, which was reduced in size. Induction of p180 led to the opposite effect, expansion of the TGN.

Cytoskeleton-linking membrane protein of 63 kDa (CLIMP-63) is the first integral membrane protein that has been reported to link an organelle, the ER, directly to microtubules (Klopfenstein *et al.*, 1998). The ER in cells overexpressing CLIMP-63 formed clusters

(Klopfensein *et al.*, 2001) or had an unusual tubular pattern (Klopfenstein *et al.*, 1998) that aligned along thickened microtubule filaments in agreement with the ability of CLIMP-63 to enhance microtubule polymerization *in vitro*. ER clusters (Klopfenstein *et al.*, 2001) or ER clustering due to retraction towards the nucleus (Vedrenne *et al.*, 2005) occurred in the absence of the microtubule binding cytosolic domain.

Interestingly, further experiments with deletion constructs have revealed that CLIMP-63 is excluded from the NE (Klopfenstein, *et al.*, 2001). This restriction is posed by the 91 nm long luminal coiled coil domain, through which the protein oligomerizes into large immobile complexes. The coiled coil domains might bind each other from the opposing ER membranes within ER lumen and thus help sheets to retain their characteristic flat shape (Klopfenstein *et al.*, 2001). While the earlier results do not point, at least very clearly, to such a role, a newly published article and earlier reviews by Shibata and collegues (2006; 2009; 2010) contain data about for example localization of CLIMP-63 to peripheral ER sheets and proliferation of ER sheets upon overexpression of CLIMP-63. However, depletion of the protein did not lead to disappearance of the sheets.

Because ER sheets and tubules have the same diameter, the highly curved sheet edges may be seen as half tubules and be formed by the membrane bending proteins (Shibata *et al.*, 2009; 2010). Indeed, a membrane bending protein reticulon 4a has been shown to localize to the edges of the flat sheet structures that form the NE in an *in vitro* assay with Xenopus membranes (Kiseleva *et al.*,2007) and all five plant reticulon-like proteins are found in the rims of peripheral ER sheets within tobacco epidermal cells (Sparkes *et al.*, 2010).

2.1.2 Fenestrated sheets

In addition to the more usually encountered ER sheet type - the intact sheet - there are quite many descriptive reports of fenestrated ER sheets (Palade, 1956; Lieberman, 1971; Orci *et al.*, 1971; 1972; Waugh *et al.*, 1973; Tani *et al.*, 1975; Brown *et al.*, 1978; Hepler, 1981; Hepler *et al.*, 1990; Novikoff *et al.*, 1983; Rambourg *et al.*, 2001). The description of the sheets in each case varies somewhat in respect to the amount of ribosomes attached as

well as to the range and mean of the fenestra size. However, apart from information that they occur across species and quite often in metabolically active tissues or cells, the purpose, biogenesis and dynamics of fenestrated sheets are completely unclear. Most likely their formation requires regulated action of membrane bending proteins within the restricted location adjacent to the molecules that keep the other portions of the sheet flat.

The *cis*- and *trans*-most cisternae of the Golgi stack appear often extensively fenestrated, and the density of fenestrations is gradually reduced towards the medial Golgi and towards the middle part of the individual cisternae (Sesso *et al.*, 1994; Ladinsky *et al*., 1999; Staehelin and Kang, 2008). Interestingly, the majority of Golgi fenestrations are small, under 65 nm in diameter (Ladinsky *et al*., 1999). The fenestra diameter in cis-cisternae peaks at about 35 nm, and gradually gets smaller towards the *trans*-side (peak at 20 nm). However, the *trans*-cisternae also include larger 65-100 nm holes. The largest holes of the cistrenae are over 100 nm in diameter. Some of the larger holes line up forming channels or wells that span through many cisternae in stacks. Although the structure of fenestrated Golgi cisternae has been known for a long time, studies pinpointing the function of fenestrations are missing.

2.1.3 Stacks of sheets

Stacks of cisternal membranes are observed both in the ER and Golgi. However, in mammalian cell culture cells the prevalent organization is different: cisternae of the ER are usually not stacked, whereas those of the Golgi are nearly always stacked.

For the Golgi, the stacking mechanism is known. The Golgi matrix proteins, Golgi reassembly stacking protein (GRASP) 65 and 55, are able to oligomerize and link adjacent Golgi cisternae together in stacks as well as laterally between stacks (Barr *et al.* 1997; Shorter *et al.*, 1999; Sengupta *et al.*, 2009). The lateral linking may also require microtubules, which the Golgi can nucleate itself (Chabin-Brion *et al.*, 2001; Wei and Seemann, 2010). The reason for cisternal stacking within the Golgi may be that it enables efficient, but sequential processing of cargo molecules. In addition to the restricted

distribution of Golgi enzymes, different morphology of the *cis*-, *medial*- and *trans*- cisternae supports the idea that functions are divided between the stacked cisternae (Dunphy and Rothman, 1985; Ladinsky *et al*., 1999). For example, the volume and the fenestration density of the cisternae in the *medial*-Golgi are smaller than in either *cis*- or *trans*-side, while the surface area is the same (Ladinsky *et al*., 1999). Finally, cisternal stacking has been shown to limit budding of vesicles from the Golgi as compared to situations, where Golgi is unstacked (Wang *et al*., 2008).

For the ER, the mechanism and reason for stacking is more unclear. Stacks of sheets are usually found in cells that secrete a lot and therefore probably represent an efficient way of organizing the membranes involved in high-rate protein synthesis (Wiest *et al*., 1990; Benyamini *et al*., 2009). However, the mechanism for stacking might be different than in the Golgi, because ER sheets in stacks are not as tightly linked as the stacked Golgi cisternae.
ER stacking proteins have not been directly shown, albeit some candidates may be found among membrane proteins that induce karmellae, stacked smooth ER cisternae, upon overexpression and/or oligomerization (Wright *et al*., 1988; Wanker *et al*., 1995; Becker *et al*., 1999; Korkhov and Zuber, 2009). Here the stacks have regular cytosolic intermembrane distance as shown by cryo-electron microscopy of vitreous sections, where karmellae were visible after overexpression of an ER transmembrane lectin chaperone, calnexin (Korkhov and Zuber, 2009). The same study also revealed the existence of ordered arrays of macromolecular complexes that spanned across both cytosolic and luminal intermembrane space. In the case of p180, the width of the cytosolic space between stacked membranes was bigger in the presence of the c-terminal domain. The c-terminus contains a coiled-coil domain, which has been shown to bind microtubules and promote their acetylation and bundling in cultured mammalian cells (Ogawa-Goto *et al*., 2007). However, it is not known whether this form of stacking is an indication of an underlying physiological phenomenon or rather an artefact or pathological condition caused by overexpression of certain transmembrane proteins (Parmley *et al*., 1976; Snapp *et al*., 2003; Lingwood *et al*., 2009). Several examples hint that the stacked karmellae-like ER does not support normal function of ER sheets. First, the exclusion of CLIMP-63 from karmellae is a sign of dissimilarity

between karmellae and normal sheets (Korkhov and Zuber, 2009). Second, the karmellae induced by interaction between overexpressed ER transmembrane protein Vap-B (vesicle associated membrane protein (VAMP) associated protein) and Nir-2 protein, attenuates protein export from ER (Amarilio *et al.*, 2005). Third, transgenic mice overexpressing a caspase-inhibitor, X-chromosome-linked inhibitor of apoptosis protein, in Purkinje cells, show neuronal degeneration and stacked ER (Korhonen *et al*., 2008). This protein is normally cytosolic, but translocates into nucleus during some stress conditions (Russell *et al*., 2008). Interestingly, the stacked ER cisternae within the Purkinje cells were tightly associated with one another and short, although all other organelles appeared normal (Korhonen *et al*., 2008). Here, the stacking was interpreted as a sign of cellular stress.

2.1.4 The nuclear envelope

The NE is the largest ER sheet in eukaryotes (Hetzer, 2010). The inner nuclear membrane (INM) has a unique set of proteins that contact the intermediate filaments within the nucleus, *i.e.* the nuclear lamina that gives stability to the nucleus, and the chromosomes. The outer nuclear membrane (ONM), that is directly continuous with peripheral ER, contains some unique proteins but exhibits also features present in peripheral ER, such as ribosomes and ER exit sites. The INM and ONM are bridged close together by luminal bridges and joined by sites containing nuclear pores.

The INM contains proteins that are immobilized by their interactions with nuclear components and may regulate gene expression (Gruenbaum *et al.*, 2005). One such protein is lamin beta receptor (LBR) that is synthesized in peripheral ER from where it diffuses to INM and binds among others to B-type lamins and DNA (Ye and Worman, 1994; Ellenberg *et al.*, 1997; Gruenbaum *et al.* 2005). The LBR sequence contains eight potential transmembrane domains, from which the first is sufficient for INM targeting (Smith and Blobel, 1993). High expression of LBR in cell culture cells induces invaginations of the NE (Ellenberg *et al.*, 1997). In neutrophils, LBR is required for creation of lobular nuclear shape, which might allow squeezing of the cells through endothelium (Hoffmann *et al.*, 2007). The 450 amino acids at the C-terminus of LBR spanning across most of the transmembrane

domains are involved in cholesterol synthesis (Waterham *et al.*, 2003; Gruenbaum *et al.*, 2005). However, a recent study shows that the first transmembrane domain in N-terminus is sufficient for the overt membrane generation observed during overexression of LBR (Ma *et al.*, 2007; Lu *et al.*, 2010).

The luminal bridges in the NE that may cause the characteristic 50 nm width of the NE are formed by transmembrane proteins containing Sad1p/UNC-84 (SUN) domains at INM and Klarsicht, ANC-1, Syne Homology (KASH) domains at ONM (Starr and Han, 2002; Wilhelmsen *et al.*, 2006; Starr and Fridolfson, 2010). These domains interact within the periplasmic space and, on the other side of the membranes, connect the NE to nuclear lamina and cytoplasmic actin, microtubule or intermediate filament cytoskeleton and to centrosomes (Starr and Fridolfson, 2010). The SUN-KASH interactions are dynamic, e.g. the proteins can change binding partners (Starr and Han, 2002), and may be modulated by the ER AAA+ ATPase Torsin A (Nery, 2008). The complexes of SUN and KASH domain proteins play roles in multiple processes including nuclear positioning, anchoring and migration, cell cycle control and apoptosis (Starr and Fridolfson, 2010). In addition, through their interactions with the cytoskeleton, they regulate positioning or structure of other organelles and may physically transduce signals from extracellular matrix directly to chromatin (Starr and Fridolfson, 2010; Jaalouk and Lammerding, 2009).

Nuclear pores are the most prominent channels within the cell: their widest diameter is 100 nm and the channel diameter 40 nm. They consist of about 30 different nucleoporin proteins (Nups) of which about half form a static scaffold that coats and may bend the NE membrane in an analogous way to for example vesicle COPII coats (Alber *et al.*, 2007). Infact, Alber and others (2007) suggest that the core structures of different vesicle coats and nuclear pores have a common evolutionary origin.

During interphase, the surface area of the NE and the number of nuclear pores increases. The extra NE membrane could be acquired from peripheral ER, since NE expansion requires connections between the two ER subdomains (Kiseleva *et al.*, 2007; Anderson and

Hetzer, 2007). Peripheral ER may have a role in nuclear pore biogenesis as well. In yeast, formation of new nuclear pores does not succeed without the membrane bending proteins, reticulon and Yop1p (Dawson *et al.*, 2009), which is surprising because they have been previously reported to be excluded from the NE (Voelz *et al.*, 2006).

2.2 Tubules and tubular networks

2.2.1 Endoplasmic reticulum tubules

Reticulons and DP1, or REEP (receptor expression enhancing protein), proteins constitute a family of ER tubule forming proteins. There are four reticulons also known as Nogo proteins, and six DP1/REEP proteins in mammals (Oertle and Schwab, 2003; Shibata *et al.*, 2009; Park *et al*., 2010). The reticulon isoforms are differentially expressed in various tissues (Oertle and Schwab, 2003). Since reticulons and DP1/REEP proteins form slow diffusing hetero- and homo-oligomers with each other, the differential expression may directly contribute to the characteristic ER morphology observed in many tissues (Shibata *et al.*, 2008; Park *et al*., 2010). The oligomerization ability is important for tubule formation, because mutants lacking the ability do not induce ER tubulation when overexpressed (Shibata *et al*., 2008). Interestingly, ATP depletion further reduces the diffusional mobilities of the oligomers, which might mean that ATP is required for disassembly of the oligomers (Shibata *et al*., 2008).

Overexpression of reticulons and DP1/REEP in yeast or mammalian cells led to altered ER morphology or tubulation and disappearance of sheets (De Craene *et al.*, 2006; Voeltz *et al.*, 2006; Park *et al*., 2010). In the case of REEP 1, microtubules were concentrated in thick bundles along aberrant tubular ER consistent with the established interaction between REEP 1 and microtubules (Park *et al*., 2010). Deletion or knockdown of reticulons and DP1 or truncation of the microtubule binding domain of REEP 1 results in the opposite morphology devoid of ER tubules (De Craene *et al.*, 2006; Voeltz *et al.*, 2006; Park *et al*., 2010).

Besides oligomerization, the membrane bending potential of reticulons and DP1/REEP proteins resides in their protein structure. They contain hydrophobic domains that may form

hairpin structures inserting to and enlarging the cytosolic membrane monolayer relative to the luminal monolayer (Voeltz *et al.*, 2006; Teng and Tang, 2008; Hu *et al.*, 2008). Hairpin-shape has been suggested to generally allow proteins to segregate into highly curved membranes (Hu *et al.*, 2009). Along with reticulons and DP1/REEP proteins, tubule formation has been shown to require a Rab GTPase, Rab-5, known for its function in endocytosis, in *C. elegans* (Audhya *et al.*, 2007). However, the exact tubule formation mechanism in this case is not known.

2.2.2 Membrane fusion and branch point formation

The Golgi and ER have a number of fusion factors that enable transport and maintain the characteristic morphology of both organelles. Fusion factors mediate both heterotypic fusion of membranes from different origins and homotypic fusion of similar membranes.

Assembly of ER or transport within Golgi is inhibited in cell free systems by treatment with N-ethyl maleimide (Glick and Rothman, 1987; Dreier and Rapoport, 2000). This is because two related ATPases, N-ethyl maleimide sensitive factor (NSF) and valosin-containing protein 97 (p97 or VCP), prime soluble NSF attachment protein receptors (SNAREs) that fuse ER and Golgi membranes (Fig. 3) in distinct ways during the cell cycle (Glick and Rothman, 1987; Rabouille *et al.*, 1995;, Warren and Malhotra, 1998; Shorter and Warren, 2002; Hetzer *et al.*, 2001; Uchiyama *et al.*, 2002; 2003; Baur *et al.*, 2007). For example, NSF is involved in heterotypic fusion of COPI-transport vesicles to target membranes during interphase. This happens by tethering the vesicles through interaction of proteinaceous linkers – Giantin in vesicular and Golgi membranes, cytosolic p115 and GM130 bound to Golgi membranes via GRASP65 - which enables binding of SNARE proteins on vesicle and target membranes and following that, fusion (Nakamura *et al.*, 1997; Seemann *et al.*, 2000; Shorter *et al.*, 2002; Ungermann and Langosch, 2005; Abdulreda *et al.*, 2009). NSF dissociates the paired SNAREs after fusion to prepare them for a new cycle of membrane fusion (Malhotra *et al.*, 1988; Barlowe, 1997).

p97 was shown to localize to ER and NE in yeast cells *in vivo* and mediate fusion of microsomes *in vitro* (Latterlich *et al.*, 1995). It is required for autophagosome biogenesis and, along with NSF, for Golgi and NE reassembly after mitosis (Rabouille *et al.*, 1995;

Kondo *et al.*, 1997; Uchiyama *et al.*, 2002; 2003; Hetzer *et al.*, 2001; Baur *et al.*, 2007; Krick et al., 2010). The substrate specificity of the two ATPases is determined by additional factors (Fig. 3). Especially p97 is known to have several activities other than membrane fusion, for example in ER associated degradation, and many of its cofactors - even the ones mediating membrane fusion - possess an ubiquitin binding domain (Meusser *et al.*, 2005; Halawani and Latterlich, 2006; Yeung *et al.*, 2008).

Closely related to ER tubule formation, the protein family of Atlastins interact with reticulons and DP1/REEP proteins and promote formation of branch points to tubular ER (Hu *et al.*, 2009; Park *et al*., 2010). *In vitro* formation of ER network from *Xenopus laevis* membranes requires atlastins and REEP proteins 1-4, as well. Atlastins are dynamin-like GTPases that have similar hydrophobic, possibly hairpin forming, membrane domains as reticulons and DP1/REEP proteins - the proteins interact through this common domain within the membrane.

At LM level, lack of functional atlastins resulted in long unbranched tubular ER structures in Hela (Rismanchi *et al.*, 2008) and COS-7 cells (Hu *et al.*, 2009). Overexpression induced sheet-like aberrant ER in COS-7 cells (Hu *et al.*, 2009). EM level inspection revealed shorter ER profiles upon depletion and expanded profiles during overexpression of the sole atlastin in Drosophila (Orso *et al.*, 2009). Depending on the isoform, atlastins are found within peripheral ER tubules and, in smaller amounts, sheets and the NE, or in cis-Golgi (Hu *et al.*, 2009; Rismanchi *et al.*, 2008; Zhu *et al.*, 2003)**.** Rismanchi and collegues (2008) reported atlastin immunogold labeling also along microtubules.

Of the other dynamin family GTPases, dynamin-1 catalyzes fission or pinching off of vesicles during endocytosis (Bashkirov *et al.*, 2008). However, atlastins are structurally more similar to mitofuzins mediating fusion of tubular mitochondria (Hermann *et al.*, 1998). Indeed, experiments with proteoliposomes by Orso *et al.*, (2009) directly show that atlastins fuse membranes *in vitro*.

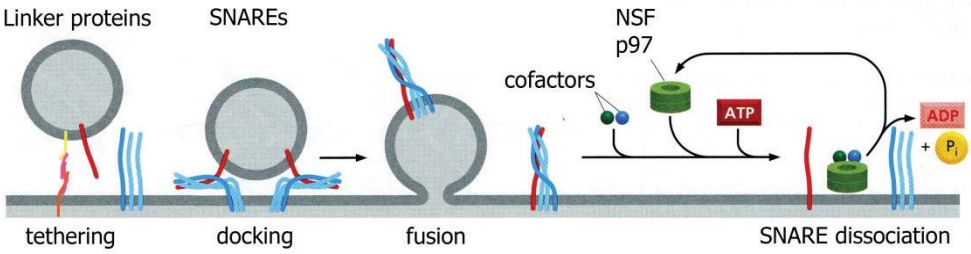

Figure 3. Membrane fusion mechanism. The image series depicts heterotypic fusion of a vesicular carrier to a target membrane, but these basic steps of membrane fusion apply to some homotypic fusion events as well. The fusion starts from binding of the membranes together through linker proteins (yellow, pink and orange), both soluble and membrane bound, that form a long tether. Tethering allows SNARE proteins (blue and red) to bind, which brings the membranes close to each other and they fuse. Two ATPases, NSF and p97, dissociate the paired SNAREs for a new round of membrane fusion. For this task, the ATPases need cofactors that for example determine the substrate specificity *i.e*. which membranes are primed for fusion. The image is derived and modified from Molecular Biology of the Cell (Alberts *et al*., 2008).

Depletion of functional atlastins disrupted morphology of the Golgi in Hela cells (Rismanchi *et al*., 2008), although not that much in COS-7 cells (Hu *et al*., 2009). The disrupted Golgi was either elongated or dispersed in morphology (Rismanchi *et al*., 2008), reminiscent of the Golgi pattern after depolymerization of microtubules (Pavelka and Ellinger, 1983; Cole *et al*., 1996). The link to microtubules may be mediated via spastin, which is a microtubule severing enzyme, and some of the REEP proteins that bind microtubules, all of which interact with atlastins (Sanderson *et al*., 2006; Park *et al*., 2010). Interestingly, mutations in atlastins, spastin and REEP proteins cause hereditary spastic paraplegias, in which long spinal neurons and their axons degenerate (Rismanchi *et al*., 2008; Hu *et al*., 2009; Park *et al*., 2010). The disease might not be linked to protein trafficking, because unlike after depolymerization of microtubules, the trafficking was unaffected in atlastin depleted cells (Rismanchi *et al*., 2008). Therefore, the conclusion is that formation of tubular ER networks and, perhaps, normal Golgi architecture are crucial for the cells, at least for neurons with long cellular extensions (Rismanchi *et al*., 2008; Hu *et al*., 2009; Park *et al*., 2010).

p22 is another protein that participates in ER branch point formation (Andrade *et al*., 2004). p22 is a cytoplasmic EF-hand Ca^{2+} binding protein capable of interacting with ER

membranes and, although not directly, with microtubules. Both ER and microtubules were disrupted by bulk microinjection of p22 antibodies, whereas microinjection of p22 protein increased the number of ER three-way junctions and microtubule bundling.

2.3 Spreading and dynamics

The cytoskeleton is required for correct localization of the ER and Golgi *i.e.* spreading of ER network and positioning of the Golgi next to centriole. Depolymerization of microtubules in mammalian cells results in slow retraction of the ER towards the nucleus (Terasaki *et al.*, 1986), collapse of ER network into cisternae (Lu *et al.*, 2009) or cisternae-like structures (Shibata *et al.*, 2008; Wozniak *et al.*, 2009). Golgi transforms into ministacks that spread throughout the cell to locations close to ER exit sites (Pavelka and Ellinger, 1983; Rogalski *et al.*, 1984; Cole *et al.*, 1996; Hammond and Glick, 2000). Cessation of ER movements has also been demonstrated (Waterman-Storer and Salmon, 1998; Wozniak *et al.*, 2009). In agreement, dynamics analysis has shown that ER tubules extend and slide along microtubules and attach to the growing microtubule tips (Lee and Chen, 1988; Waterman-Storer and Salmon, 1998; Wozniak *et al.*, 2009). Membrane tubules can be pulled out from membranes with the help of molecular motors moving along microtubules (Roux *et al.*, 2002). A recent study revealed that ER tubules extend away from the nucleus with the help of kinesin-1 and towards the nucleus with the help of dynein (Wozniak *et al.*, 2009). Inhibition of both motors induced a shift from tubular to lamellar morphology as evaluated at LM level. The pericentriolar position of the Golgi is maintained by dynein motors (Corthesy-Theulaz *et al.*, 1992). Nuclear positioning also depends on microtubules (Roux *et al.*, 2009; Starr and Fridolfson, 2010). Contrary to tubules and even the NE, dynamics of ER sheets is an unexplored field. This is presumably because the movements are best resolved in the thin lamellipodia with a single peripheral layer of ER and the more usual localization of sheets in mammalian cell culture cells is perinuclear.

Some of the ER proteins behind microtubule mediated ER movements have been identified. For example, stromal interaction molecule 1 (STIM-1), has been shown to mediate ER tubule extension by attaching ER to the tip of a growing microtubule (Grigoriev *et al.*, 2008). The roles of p180 and CLIMP-63, the proteins that might contribute to formation of sheet

structures, in ER spreading are indicated by microtubule binding domain deletion and knockdown experiments during which ER retracts towards the cell center (Vedrenne *et al.*, 2005; Ogawa-Goto *et al.*, 2007).

Depolymerization of the actin cytoskeleton, on the other hand, does not have such an obvious effect on ER structure or dynamics, at least not in some mammalian cells (Chhabra *et al.*, 2009; Wozniak *et al.*, 2009). However, some indications of interactions between actin and ER exist. First, ER vesicles and tubules are transported along actin filaments in neuronal cells with the help of Myosin V motor (Tabb *et al.*, 1998; Langford, 1999). Second, cytochalasin D inhibits slow retrograde flow of the ER towards the cell center (Waterman-Storer and Salmon, 1998). Third, links between peripheral ER and actin are provided via an ER protein inverted formin 2 (Chhabra *et al.*, 2009) that accelerates both actin polymerization and depolymerization, as well as the ER calcium channel, testosterone-repressed prostate protein-2 (TRPP-2), that protects cells from apoptosis (Chen *et al.*, 2008; Wegierski *et al.*, 2009). Interestingly, TRPP-2 interacts directly or indirectly with other proteins that have established or potential ER shaping functions: kinesins, the fusion/ER associated degradation factor p97 and intermediate filaments (Chen *et al.*, 2008).

Despite the clear effect of cytoskeleton on ER network spreading, several experiments show that cytoskeleton is not solely responsible for it. Yip1a is a membrane protein that cycles between the ER and *cis*-Golgi (Dykstra *et al.*, 2010). Its depletion leads to formation of stacked and whorled ER membranes and slowing of the COPII mediated transport without notable changes in the microtubule cytoskeleton. Futhermore, ER networks can be produced from Xenopus egg membrane fractions (Dreier and Rapoport, 2000) and rat hepatocyte microsomes (Lavoie *et al.*, 1996) without inclusion of cytosol, the reservoir for cytoskeletal components.

3. Function and form: rough and smooth endoplasmic reticulum and Ca^{2+}

The functions of both ER and Golgi are multiple and diverse. Some functions are better done in a sequential way, while others may hinder or even compete with each other requiring some efficient means of separation. The structure-function relationships within the ER network are not very clear and are difficult to study, since boundaries are hard to define within a network in which all subdomains – the rough ER (RER), the smooth ER (SER) and the NE – are directly continuous with each other. On the other hand, organization of different functions in structural subdomains of the Golgi is well characterized (Dunphy and Rothman, 1985) and further studied (Emr *et al*., 2009) and therefore I will not concentrate on them here.

3.1 Segregation and morphology of rough and smooth endoplasmic reticulum

Peripheral ER is classically divided by its appearance in EM to ribosome containing RER and to SER devoid of ribosomes. By screening through different cell types, it has been concluded that cells engaged in high-rate protein synthesis or secretion have a lot of RER organized as sheets, which may be stacked (Wiest *et al.*, 1990; Rajasekaran *et al.*, 1993; Benyamini *et al.*, 2009; Ueno *et al.*, 2010a). In these cells, the Golgi is large meaning that there are many cisternal stacks and vesicles, cisternae are long or TGN reaches towards the periphery of the cell (Griffiths *et al.*, 1989; Wiest *et al*., 1990; Benyamini *et al.*, 2008; Ueno *et al.*, 2010b). Cells involved in lipid and steroid synthesis and detoxification, on the other hand, have abundant tubular SER (Black *et al.*, 2005).

The ribosome is big machinery, about 5000 kDa, consisting of a large and a small subunit that synthesize proteins according to mRNA templates (Ramakrishnan, 2002). However, the size of the assemblage engaged in protein synthesis is still a lot bigger as presented by Nikonov and Kreibich (2003). Protein synthesis requires factors targeting mRNAs and ribosomes to ER, the translocon channel for binding the ribosome and translocating the nascent polypeptide through the ER membrane and the luminal machinery consisting of chaperones, glycosylation and other modifying enzymes. Given that mRNA weighs 500 kDa

per each kb and many ribosomes can be translating the same mRNA, *i.e.* the polysome**,** the total molecular weight reaches easily several tens of thousands kDa. Not surprisingly, the lateral diffusion speed of translocons that are bound to polysomes is very low (Nikonov *et al.*, 2002; 2007). This is in contrast to RER proteins that are not part of polysomal assemblies (Rolls *et al.*, 2002). The diffusion speed of polysomes is dependent on intact microtubule filaments or their linkage to ER, since depolymerization of microtubules, depletion of the immobile ER protein CLIMP-63 or deletion of its microtubule binding domain, resulted in faster diffusion (Nikonov and Kreibich, 2003; 2007). Depolymerization of actin filaments did not affect the diffusion speed. This led to the proposal that RER domains with polysomes could be fenced by microtubules anchored to ER.

Interestingly, p180 binds both microtubules and ribosomes on ER (Ogawa-Goto *et al.*,2007; Bai *et al.*, 2008), and when expressed in high amounts, induces RER formation and enhanced secretion in some cells types (Benyamini *et al.*, 2009; Ueno *et al.*, 2010a). The structural identity of the RER formed has not been studied in 3D, but long cross-sectional profiles in EM images imply sheet morphology. In addition, it has been postulated that large polysomes may not fit into highly curved tubular membranes (Shibata *et al.*, 2006). In agreement, the mean diameter of rough microsomes extracted from rat hepatocytes is larger (120 nm) than that of smooth microsomes (80 nm) (Lavoie *et al.*, 1996). Similarly to CLIMP-63 and polysomes, the membrane bending proteins are organized as immobile oligomers in tubules (Shibata *et al.*, 2008), suggesting that restricted diffusion and differential distribution of proteins according to membrane curvature may keep RER and SER domains apart.

3.2 Structure-function relationship of rough and smooth endoplasmic reticulum

What is the advantage in building RER sheets and SER tubules? These shapes have probably formed to best fulfill their functions. Membrane-coupled and luminal reactions require a large surface area and luminal volume, respectively. The surface area to volume ratio is quite high in small transport vesicles as well as tubules as opposed to planar sheets (Otegui *et al.*, 2001). In accordance with this idea, the functions of tubules seem to be linked to lipid synthesis etc. requiring and producing a lot of membrane surface. Similarly, the

sheets have been proposed to offer a more efficient platform for protein synthesis and modification – which requires a large luminal machinery – than tubules (Rajasekaran *et al.*, 1993). For convoluted sheets or fenestrated sheets with a small fenestration diameter, the question is more complicated, because the surface to volume ratio increases (Otegui *et al.*, 2001) and it is not known whether their function somehow correlates with this increase.
The straightforward idea that RER corresponds to flat sheets and SER to tubules is hampered by frequent reports mentioning RER tubules, about fenestrated sheets containing high curvature membranes and varying amount of ribosomes (Palade, 1956; Orci *et al.*, 1971; 1972; Waugh *et al.*, 1973; Tani *et al.*, 1975; Lieberman, 1971; Brown *et al.*, 1978; Hepler, 1981; Hepler *et al.*, 1990; Novikoff *et al.*, 1983; Rambourg *et al.*, 2001), or artificially induced smooth sheets (Wright *et al.*, 1988; Wanker *et al.*, 1995; Becker *et al.*, 1999; Lingwood *et al.*, 2009) and functional RER components found in SER (Black *et al.*, 2005). Futhermore, the exact mechanism keeping the sheet membranes flat with the characteristic spacing of about 100 nm, has not been elucidated.

Transformations of sheets to tubules or *vice versa* should coincide with changes in ER functions. Sheet-to-tubule transformations have been documented in plants (Quader and Zachariadis, 2006) at low temperature and intracellular pH (<6,8), and might reflect inhibition of protein synthesis and transport occurring under these conditions (Cosens *et al.*, 1976; Saraste and Kuismanen, 1984; Cosson *et al.*, 1989; Sugden and Fuller, 1991; Sephton and Driedzic, 1995). Transformation from tubules to sheets takes place at high >35˚C temperature or pH (>7,5), pathogen attack, dehydration and after perturbation of Ca^{2+} distribution (Quader and Zachariadis, 2006). Here, the morphological change might be induced by various reasons including increased protein synthesis (Sugden and Fuller, 1991; Sephton and Driedzic, 1995) or natural stress response, the unfolded protein response, which upregulates ER associated degradation, production of additional folding enzymes to ER lumen, lipid biosynthesis and proliferation of ER sheets (Schuck *et al.*, 2009).
However, contrary to this putative coupling of function to form, Schuck and collegues (2009) show their uncoupling in yeast. They stressed the cells to induce the unfolded protein response and, consequently, ER sheets. Next, the same was done during overexpression of

reticulon proteins, which converted the sheets to tubules. Interestingly, the tubular morphology did not diminish the ability of the cells to manage the stress. The authors concluded that the membrane expansion itself, which happened by upregulation of lipid synthesis, was sufficient to alleviate the stress.

3.3 Regulation of endoplasmic reticulum shape and dynamics by Ca^{2+}

Ca^{2+} is an important regulator of many proteins and functions within cells. It is required for example in protein folding, regulation of cell division and apoptosis (Giorgi *et al.*, 2009). Both ER and Golgi are Ca^{2+} stores within cells: the distribution of the ion appears more homogenous within the Golgi than ER (Pezzati *et al.*, 1997). Correct Ca^{2+} concentration is important for regulation of different functions. For example, Ca^{2+} efflux from Golgi controls intra-Golgi vesicular transport: it stimulates it at low (20-100 nM) and inhibits it at high (>100 nM) concentration (Porat and Elazar, 2000).

Correct Ca^{2+} concentration is also important for the morphology of the organelles. p22 is for example a protein, which responds to Ca^{2+} and shapes ER membranes (Andrade *et al.*, 2004). Ca^{2+} induces a conformational change in the protein increasing its binding to microsomes. Interestingly, this is important for branch point generation: microinjection of wild type (wt) p22 into cells increases the number of branch points within ER, which is not seen after microinjection of a mutant p22 that cannot change conformation in response to Ca^{2+}.

Some Ca^{2+} in the cytoplasm is clearly required for ER network formation, since Ca^{2+} is released from the ER itself during the process in cell free network assembly experiments (Voeltz *et al.*, 2006). With the same system, Dreier and Rapoport (2000) have shown that both chelation of Ca^{2+} or high Ca^{2+} concentration inhibits ER network formation, but not the basic fusion reaction. High Ca^{2+} concentration that was induced for example by ionomycin, caused vesiculation of ER within cells and *in vitro*, although in the latter case the vesicles were often still tethered together.

However, ER fragmentation is not directly linked to increased cytosolic Ca^{2+} concentration in a number of studies. First, in human embryonic kidney 293 cells, a treatment with ionomycin induced formation of punctate structures at LM level, which appeared to correspond to

branched but not fragmented RER structures at EM (Ribeiro *et al.*, 2000). Second, in neurons, increased Ca^{2+} concentration seemed to fragment ER membrane, but the effect was blocked by N-methyl, D-aspartate receptor agonist independent of the Ca^{2+} levels (Kucharz *et al.*, 2009). Third, thapsigargin is a drug that depletes Ca^{2+} stores within ER and increases the cytosolic Ca^{2+} concentration. During thapsigargin treatment, the ER protein STIM-1 oligomerizes and relocalizes in punctate structures within ER near the plasma membrane (Smyth *et al.*, 2009). There it interacts with and activates store operated Ca^{2+} channels of the plasma membrane: influx of Ca^{2+} through the channels follows. At EM level, STIM-1 is observed in ER sheets aligning along the plasma membrane (Orci *et al.*, 2009). There are thin sheets completely devoid of ribosomes and thicker ones with ribosomes only on the side facing cytosol.

The ER contains many channels for Ca^{2+} intake or release including sarco/endoplasmic reticulum Ca^{2+} -ATPase, inositol 1,4,5-trisphosphate receptor, ryanodine receptor and TRPP-2 (Taylor *et al.*, 2009). However, there is an ongoing debate whether some Ca^{2+} is released through translocation channels after completion of protein synthesis under physiological conditions (Roy and Wonderlin, 2003; Van Coppenolle *et al.*, 2004; Ong *et al.*, 2007; Amer *et al.*, 2009). Ca^{2+} release through the translocons happens for example when cells are treated with puromycin (Van Coppenolle *et al.*, 2004; Ong *et al.*, 2007). Puromycin is an inhibitor of translation that induces premature chain termination and splits the ribosome in two parts. Puromycin treatment leaves some ribosomes on ER (Seiser and Nicchitta, 2000) and Ca^{2+} leaks out through the translocons that are bound only by the large ribosomal subunit (Roy and Wonderlin, 2003). Another inhibitor of translation, cycloheximide, does not induce Ca^{2+} leak through the translocon or generally, reduction in ER calcium strores (Roy and Wonderlin, 2003; Van Coppenolle *et al.*, 2004). Cycloheximide blocks elongation of the peptide chain and does not split the ribosome and release polysomes from ER (Roy and Wonderlin, 2003, Van Coppenolle *et al.*, 2004; Seiser and Nicchita, 2000). Even a long treatment with cycloheximide did not induce Ca^{2+} leakage, although it stripped some ribosomes from ER membranes (Van Coppenolle *et al.*, 2004).

ER movement is halted in parallel with mitochondrial movement in response to high cytosolic Ca^{2+} concentration (Brough *et al.*, 2005). ER and mitochondria have multiple contact sites, where lipids are transferred between the organelles, and Ca^{2+} signals released from ER and taken in by mitochondria to regulate for example ATP synthesis and survival (Giorgi *et al.*, 2009). High cytosolic Ca^{2+} concentration has been shown to lead to tighter association of the organelles, while low concentration dissociates them (Wang *et al.*, 2000). Electron tomographic reconstructions have shown that ER and mitochondria are joined by tethers that are shortened in response to apoptotic drugs (Csordas *et al.*, 2006). When tethers were shortened artificially, the mitochondria were easily overloaded with Ca^{2+}, which promotes apoptosis. The normal tether lengths were 10 nm at SER and 25 nm at RER. The coupling of RER membranes to mitochondria is, however, not reported by Wang and others (2000), who described the ER associated with mitochondria as SER.

4. Membrane shaping during mitosis

4.1 Breakdown and reassembly of the Golgi

The mitotic breakdown of the mammalian Golgi apparatus into dispersed tubulo-vesicular clusters has been studied in detail (reviewed by Shorter and Warren, 2002) and is important for mitotic entry (Sutterlin *et al.*, 2002; Hidalgo Carcedo *et al.*, 2004; Rabouille and Kondylis, 2007). The Golgi fragments during late G2 to prometaphase through COPI dependent and independent routes (Misteli and Warren, 1995a and b; Feinstein *et al.*, 2007; Hidalgo Carcedo *et al.*, 2004). In both pathways, the mechanism behind is continued membrane fission coinciding with inhibited fusion and stacking through phosphorylation of fusion factors and structural proteins. For example, COPI vesicle docking and fusion is inhibited through changes in the phosphorylation state of GM130 (Lowe *et al.*, 2000) and p115 (Sohda *et al*., 1998), whereas the vesicle budding goes on and even increases (Sönnichsen *et al.*, 1996; Wang *et al.*, 2008).

The Golgi is disassembled in discreet steps: tubular connections between cisternal stacks are severed first, cisternae are unstacked, vesiculation may disassemble most of the cisternal edges and the rest is transformed into tubulovesicular fragments (Misteli and

Warren, 1994; 1995a and b; Sönnichsen, 1996; Barr *et al.*, 1997; Hidalgo Carcedo *et al.*, 2004). Intermediates, such as fenestrated sheets and tubular networks, are observed and act as substrates for further fragmentation. Fission mediated by c-terminus binding protein 3/brefelding A adenosine diphosphate-ribosylated substrate has been shown to fragment already tubulated Golgi membranes into tubulovesicular clusters (Hidalgo Carcedo *et al.*, 2004). Golgi disassembly shows large cell-type specific variation in the extent of fragmentation. Specifically, in some cell types the fragmentation yields vesicular and tubular products (Lucocq *et al.*, 1987; Jokitalo *et al.*, 2001; Seemann *et al.*, 2002), whereas tubules or cisternae are found in others (Maul and Brinkley, 1970; Moskalewski, 1977; Moskalewski and Thyberg, 1990).

Many Golgi factors that are liberated during disassembly show altered localizations within the cell that may be associated with special mitotic functions of the proteins (Wei and Seemann, 2010). For instance, Nir-2, a peripheral Golgi/ER protein, is mitotically phosphorylated after which it shows cytosolic localization distinct from the mitotic Golgi membranes (Litvak *et al.*, 2004). In anaphase it redistributes to the cleavage furrow, where it regulates cytokinesis.

Reassembly of the Golgi takes place in telophase. Here, fusion activities by NSF and p97 resume (Fig. 3; Rabouille *et al.*, 1995). Interestingly, p97 has a cofactor specialized in the reassembly events: p47 (Kondo *et al.*, 1997; Uchiyama *et al.*, 2002; 2003). It mediates binding of p97 to the target SNARE on Golgi, syntaxin 5, (Rabouille *et al.*, 1998), after which another cofactor, VCP[p97]/p47 complex-interacting protein, p135 (VCIP135) binds and enables dissociation of the complex through p97 catalyzed ATP hydrolysis. The regulation of p47 is ingenious: during interphase it is kept within the nucleus away from its substrates residing in the cytoplasm and is then released from the nucleus during nuclear envelope break down (NEBD) (Uchiyama *et al.*, 2003). However, its binding to Golgi membranes is suppressed during mitosis through phosphorylation by cell division control protein 2 homolog (Cdc-2), and if the suppression is lacking, Golgi fails to disassemble. Phosphorylation is removed in telophase, possibly because myelin transcription factor 1

suppresses Cdc-2 kinase activity (Nakajima *et al.*, 2008), and Golgi reassembly starts (Uchiyama *et al.*, 2003).

4.2 Reshaping the endoplasmic reticulum and Golgi

4.2.1 Common morphogenetic mechanisms

Many factors and mechanisms, most notably those involving phosphorylation and inhibition of fusion, mediating dis- and reassembly of the Golgi, affect ER structure during mitosis. First, in the open mitosis of mammalian cells, the NE is disassembled to allow interactions between spindle microtubules and chromosomes. Many NE proteins are phosphorylated mitotically, which interferes with their binding interactions with the interphase partners. For example, LBR becomes phosphorylated by Cdc-2 kinase (Courvalin *et al.*, 1992). Its binding partner, lamin B, is phosphorylated by beta II protein kinase C (Goss *et al.*, 1994). Like Golgi proteins, some components of the nuclear pores have special mitotic functions (Hetzer, 2010).

Second, interactions between some peripheral ER components have been shown to be disrupted by phosphorylation during mitosis. An analogy might be drawn between the Golgi stacking protein GRASP-65, which undergoes mitotic phosphorylation leading to disruption of its homo-oligomerization and consequently, unstacking of the Golgi (Barr *et al.*, 1997), and for example Nir-2 on ER. Nir-2 is a karmellae forming protein that is phosphorylated and shows an altered localization during mitosis (Amarilio *et al.*, 2005; Litvak *et al.*, 2004).

Third, Dreier and Rapoport (2000) provide data implying that the mitotic kinase Cdc-2 inhibits ER fusion during mitosis. They detected significantly less three-way junctions in the ER network assembly assay, when they used extracts with constitutively active Cdc-2. Consequently, it was shown that removal of Cdc-2 kinase activity by myelin transcription factor 1 and therefore dephosphorylation of several mitotic substrates in telophase, is important for correct reassembly of the ER *in vivo* (Nakajima *et al.*, 2008). Importantly, p97/p47 gains its fusion activity in late mitosis and restores ER structure back to interphase morphology (Uchiyama *et al.*, 2002; 2003). Inhibition of this system, the same system as in Golgi reassembly, p97/p47, syntaxin-5 on ER and VCIP135, led to decreased numbers of

ER three-way junctions, as quantified from post mitotic cells that were imaged with confocal microscope *in vivo* (Uchiyama *et al.*, 2002). The NE assembly also uses the fusion power of p97/p47, but requires in addition NSF (Baur *et al.*, 2007) and another p97 cofactor, the Ufd1–Npl4 complex (Hetzer, 2001).

4.2.2 Mitotic structure of the peripheral endoplasmic reticulum

Despite many similarities between Golgi and ER partitioning, there are some notable differences in respect to membrane fission and structures. Unlike COPI, COPII vesicle budding from the ER is blocked, because ER exit sites are disassembled during mitosis (Farmaki *et al.*, 1999; Prescott *et al.*, 2001). Therefore, the ER may not be consumed by continuous vesicle fission events. This is reflected in the different localizations of the ER and Golgi during mitosis: in fluorescent images the ER usually resides quite homogenously around the spindle of the cell, while the Golgi forms small and scattered punctae in addition to a more uniformly distributed haze (Fig. 4). The mechanisms to obtain equal partitioning of ER and Golgi membranes might therefore be different.

Little is known about the final ER structure that mediates partitioning to daughter cells or if a common structure even exists. It seems that morphology of mitotic ER, like Golgi structures, varies considerably between cells of different species, cell types and developmental stages.

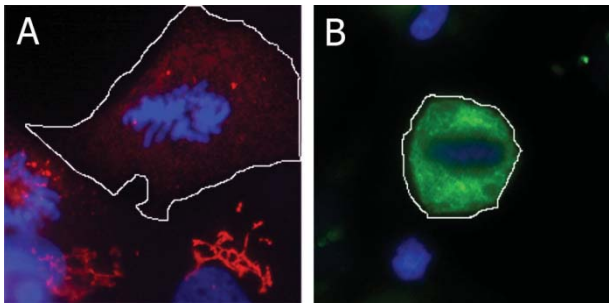

Figure 4. ER and Golgi localizations differ during mitosis. (A) Golgi in a mitotic NRK-52E cell was stained with an antibody against the cis-Golgi marker GM130 and shows scattered small dots and haze. Cells on the upper left and middle are in early and late prometaphase, respectively. (B) ER in a metaphase Huh-7 cell is distributed evenly around the chromosomes and spindle. Images were acquired with a wide-field microcope. DNA was stained with DAPI in both images.

Early EM studies based on observation of thin sections of mammalian cells (Zeligs and Wolmann, 1979; Tamaki *et al*., 1991) and the analysis of the ER in cell free systems suggested fragmentation of the ER (Collas and Courvalin, 2000), at least to variable extent. Observations about fenestrated sheets in mitotic Hela cells (Mullins, 1984) and plants (Hepler, 1980; Hawes *et al.*, 1981) were also published. Later, measurements of diffusion within mitotic ER in mammalian cells (Ellenberg *et al.*, 1997) or sea urchin embryos (Terasaki *et al.*, 2000) indicated that the continuity of the ER is retained. Another study of Hela cells concluded that the ER is highly interconnected and almost exclusively cisternal in form (McCullough and Lucocq, 2005). Whatever the mitotic structure, distribution of ER has been agreed about in several studies: it is mostly excluded from the spindle region (Ellenberg *et al*., 1997; McCullough and Lucocq, 2005; Parry *et al*., 2005).

The role of membrane bending proteins in restructuring mitotic ER has been studied in *C. elegans* (Aydhya *et al.*, 2007). There, ER forms mitotic clusters composed of tightly packed thick tubules. ER morphology in *C. elegans* depleted of YOP-1 and RET-1, homologues of DP-1 and reticulon 4a, was devoid of these clusters during mitosis and reduced the viability of *C. elegans* embryos by 60%. Interestingly, in the same study, expression of a mutant Rab-5 that is slow in GTP hydrolysis, induced formation of multiple mitotic ER clusters even in HeLa cells (Audhya *et al.*, 2007).

Related to membrane curvature, Liu and Zheng (2009) recently showed that mitotic ER in HeLa cells exhibits long, stacked and clustered ER membranes upon depletion of Epsin, which is an endocytic adapter protein during interphase. The cells had also problems in spindle morphogenesis. It appeared that the membrane bending ENTH domain of Epsin was responsible for the normal appearance of mitotic ER, although the subcellular localization of Epsin during mitosis is not known. Nevertheless, the authors propose that a highly interconnected membrane network provides elastic support for the spindle, implying that bending of mitotic ER membranes is required for correct cell division.

The putative linkage between polysomes/RER and sheet morphology leads to the obvious question: what happens to ribosomes during mitosis? It has been known for a long time that protein synthesis is downregulated during mitosis and polysomes may or may not break

down (Scharff and Robbins, 1966; Steward *et al.*, 1968; Le Breton *et al.*, 2005; Sivan *et al.*, 2007). Disassembly or conservation of polysomes might depend on the inhibition mechanism of translation: inhibition of initiation would lead to disassembly, whereas inhibition of elongation causes conservation as shown in mitotic Hela cells (Sivan *et al.*, 2007).

Transient or sustained Ca^{2+} signals have been shown to be important for mitotic progression (Ratan *et al.*, 1988; Tombes and Borisy, 1989; Parry *et al.*, 2005). At least in one case, Ca^{2+} binding within ER has been directly shown to regulate cell division. In mammalian cells, the Tweety homologue 1 is a transmembrane ER protein implicated in Ca^{2+} binding and ion channel activity (Kumada *et al.*, 2010). It is highly expressed in terminally differentiated neural cells as well as in some proliferating embryonic neural cells and its disruption leads to embryonic lethality in mouse. The knockout cells fail to divide normally in culture, suggesting a role as a mitotic regulator. Unfortunately, reports that would directly describe the effect of Ca^{2+} on ER morphology during mitosis in mammalian cells are missing.

4.2.3 Nuclear envelope breakdown and reassembly

The best studied ER subdomain during mitosis is no doubt the NE. Its disassembly takes place in prophase and begins by loss of some Nups from the nuclear pores (Ellenberg *et al.*, 1997; Lénárt *et al.*, 2003). Although all Nups are displaced, some are retained in subcomplexes during cell division and then recruited back to the forming NE (Doucet *et al.*, 2010). In general, membrane bound NE proteins seem to diffuse to peripheral ER (Yang *et al.*,1997; Ellenberg *et al.*, 1997), whereas soluble NE proteins can be cytosolic or associate with for example kinetochores, spindle poles or microtubules, where they affect for example spindle organization (Dechat *et al.*, 2004; Hetzer, 2010). Interestingly, the nucleoporin Nup153 has been shown to participate in NEBD and interact with COPI complex directing it to the NE (Liu *et al.*, 2003). However, the role of COPI at mitotic NE is not known. NEBD, although not chromosome condensation, is delayed when the *C. elegans* YOP-1 and RET-1 proteins are depleted suggesting that membrane bending promotes NEBD (Aydhya *et al.*, 2007).

ER tubule ends bind to chromatin and start the NE reassembly process both *in vitro* and *in vivo* (Anderson and Hetzer, 2007; 2008). LBR, which binds to chromatin, has been shown to play an essential role in targeting the precursor NE membranes to chromatin in late anaphase to telophase (Collas *et al.*, 1996; Ellenberg *et al.*, 1997). A recent report reveals that the recruitment of LBR is mediated by importin β (Lu *et al.*, 2010). This interaction is dependent on phosphorylation of LBR by Cdc-2 and is required for NE assembly. The targeting happens concomitantly with the assembly of nuclear pores (Hetzer, 2010).

Cell-free studies indicate that the NE formation proceeds by creation of a tubular network with small flat patches or sheets on chromatin surface (Dreier and Rapoport, 2000; Anderson and Hetzer, 2007). Next, the patches enlarge over time and finally seal the nucleus. This stage involves fusion reactions mediated by NSF, without which the NE does not flatten (Baur *et al.*, 2007), and the p97/Ufd1–Npl4 complex (Hetzer *et al.*, 2001). The subsequent NE growth is mediated by p97/p47. Interestingly, according to Anderson and Hetzer (2008), endogenous levels of membrane bending proteins that are excluded from the NE, are rate limiting to the mammalian NE formation *in vivo*. However, a study of NE growth in Xenopus egg extracts shows that reticulon 4a localizes to connections between the NE and peripheral ER as well as to the edges of flattening membranes on chromatin (Kiseleva *et al.*, 2007). Antibody to reticulon 4a induced a block in the growth phase of the sealed NEs (Kiseleva *et al.*, 2007) and so did destroying the NE-peripheral ER connections by mechanical stress (Anderson and Hetzer, 2007), which suggests that the curved connections feed membrane from the periphery into the NE.

4.2.4 Cytoskeleton during mitosis

In mitosis, the microtubules form the mitotic spindle and many, but not all, of the microtubule dependent membrane movements are inhibited (Warren, 1993; Niclas *et al*., 1996; Shima *et al.*, 1998; Farmaki *et al*., 1999). The membranous end products of Golgi disassembly, mitotic Golgi clusters and vesicles, associate with spindle poles and astral microtubules and are evenly divided between the daughter cells already before metaphase (Shima *et al.*, 1998; Jokitalo *et al.*, 2001). Motor protein mediated movements of the NE along microtubules may be a mechanism that ruptures the NE (Beaudouin *et al.*, 2002), although

NEBD has been shown to occur without microtubules *in vitro* (Lénárt *et al.*, 2003). Otherwise the extent and mediators of ER movements during mitosis are unknown.

Several interphase associations of the ER to the microtubule cytoskeleton are apparently lost during mitosis through down regulation of specific proteins or phosphorylation, which occurs for example to the putative sheet proteins p180 (Benyamini *et al.*, 2009) and CLIMP-63 (Vedrenne *et al.*, 2005), respectively. In addition, STIM-1 localizes to ER sheets near the plasma membrane and enables store operated Ca^{2+} entry (Orci *et al.*, 2009) and mediates ER tubule extension via the tip attachment mechanism in interphase (Grigoriev *et al.*, 2008). However, in mitotic cells STIM-1 becomes phosphorylated, cannot bind microtubules and loses its association with plasma membrane (Smyth *et al.*, 2009).

There are some indications that the actin cytoskeleton, located in the cell cortex near the plasma membrane, would take over the task of microtubules during mitosis. First, Wollert and collegues (2002) provided evidence that in metaphase Xenopus egg extracts the ER moved and build networks on F-actin. The motility was dependent on Myosin V motor. Second, mitotic ER in Hela cells was found in close association with cortical actin. (McCullough and Lucocq, 2005). Depolymerisation of actin filaments with latrunculin A caused ER to retract towards the spindle and change shape from cisternal to a more tubular form.

Aims of the study

The aims of this study were to reveal structures and mechanisms - the morphogenesis - of the ER and Golgi apparatus with particular attention on changes occurring during mitosis. Specifically, the aims were

1. To elucidate what kind of structural changes occur in the ER of mammalian cells during mitosis.
2. To determine whether and in what quantity ribosomes associate with different types of high and low curvature ER membranes and could ER-bound ribosomes stabilize sheets.
3. To analyse how fenestrated ER sheets differ from intact sheets or what could be the function of fenestrated sheets.
4. To study the *in vivo* role of the fusion factor p97/p37 in the maintenance and morhogenesis of the Golgi apparatus and ER.

Methods

The methods used in this thesis are listed below with the reference to the original publication. A detailed description of the methods not included in the original publications is found after the table.

Method	**Publication**		
Cell Culture			
CHO-K1	**I**		
CHO-K1/Hsp47-GFP	**I**		**III***
HeLa		**II**	**III***
Huh-7		**II**	
NRK			**III***
NRK-52E		**II**	
Vero		**II**	
Construction and culture of cell lines			
CHO-K1/LBR-GFP	**I**		
CHO-K1/LBR-HRP	**I**		
CHO-K1/ssGFP-KDEL	**I**	**II**	
CHO-K1/ssHRP-KDEL	**I**		
HeLa/LBR-GFP		**II**	
HeLa/LBR-HRP		**II**	
Manipulation of cells			
Single plasmid transfections	**I**	**II**	**III***
Cotransfections		**II**	
Cycloheximide treatment	**I**		
Puromycin treatment	**I**		
Nocodazole treatment		**II**	
Latrunculin A treatment		**II***	

siRNA treatments			III*
Microinjections			III*
Proteasome inhibitor treatment			III*
Sample preparation for microscopy			
Immunofluorescence staining	I		III*
Cytochemical staining	I	II	
High pressure freezing/Freeze substitution		II	
Flat embedding	I	II	
Embedding cell pellets		II	
Sectioning	I	II	
Microscopy			
Wide field microscopy	I	II	
Laser scanning confocal microscopy	I	II	III*
Electron tomography	I*	II*	
Transmission electron microscopy	I	II	III
Correlative light electron microscopy		II	
Image analysis			
ImagePro	I	II	
TILL VisION	I	II	
AutoQuant	I	II	
Imaris Bitplane	I	II	
Aida Image Analyzer	I		
NIH Image			III*
MatLab		II**	
Amira	I**	II	
Imod	I**	II	
VCell		II*	
DNA methods			
Construction of pLBR-HRP	I		

Construction of pssGFP-KDEL	**I**		
Construction of p97			**III***
Construction of p37			**III***
Polymerase chain reaction	**I**		**III***
Protein methods			
Western blotting analysis	**I**		**III***
Amino acid sequence alignments			**III***
Yeast two-hybrid screen			**III***
Immunoprecipitation			**III***
Protein expression and purification			**III***
Production of antibodies			**III***
Manipulation of organelles *in vitro*			
Subcellular fractionation			**III***
In vitro Golgi reassembly assay			**III***
Quantification from images			
Ribosomes	**I**	**II**	
ER structures	**I**	**II**	**III***
ER dynamics		**II****	
In vivo Golgi structures			**III**
In vitro Golgi structures			**III***
Western blot band densities	**I**		**III***
Statistical analyses	**I**	**II**	**III***

**Method not applied by the author

**Method in which the author had minor contribution

Construction of pTREssHRP-KDEL/CHO-K1 cell line (I)

pTREssHRP-KDEL was created by polymerase chain reaction amplification of SacII-kozak sequence-signal sequence-horse radish peroxidase-KDEL-BamHI from the pssHRP-KDEL (Connolly *et al.*, 1994) and cloning it into pTRE backbone (Clontech). The stable cell line expressing ssHRP-KDEL was created according to Tet-Off Gene expression system guide lines (Clontech). Briefly, the CHO-K1 wt cells were first stably transfected with pTet-Off and the selected clone then cotransfected with pTK-Hyg and pTREssHRP-KDEL. The resulting clones were screened for expression of the ssHRP-KDEL and a clone with moderate expression level selected. Despite the regulatory Tet-Off system, the expression of the clone used (I) did not respond to regulation by doxycyclin.

GM130 immunostaining

Cells for fluorescence immunolabelling were fixed with 4% PFA, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$ for 15 min, quenched with 0.12% glycine for 10 min and permeabilized with 0.1% Triton-X 100 for 4 min, all in PBS. Blocking, mouse anti-GM130 (Transduction Laboratories) and secondary antibody (anti-mouse rhodamine red-X, Jackson Immunoresearch) stainings were done in 1% BSA in PBS for 30 min each. DNA was stained with 1 µg/ml DAPI (Roche) in PBS for 5 min before mounting in Mowiol (Hoechst) supplemented with DABCO antifading reagent (Sigma).

Results

1. Interphase architecture of the endoplasmic reticulum and Golgi

1.1 Interphase architecture and dynamics of the endoplasmic reticulum varies between different cell types

1.1.1 Position and abundance of different endoplasmic reticulum structures is variable (I; II)

Interphase ER in mammalian cell culture cells typically consist of a network that is more enriched in sheets near the nucleus, whereas peripheral regions have tubules (Terasaki *et al.*, 1986). Using LM, we observed this typical organization in CHO-K1 (I: Fig. 1A), HeLa and Vero (II: Fig. 2A) cells expressing ER markers coupled to green fluorescent protein (GFP). Thin EM sections of wt cells or cells with an ER marking cytochemical staining confirmed the result (I: Fig. 1B). Further, we modeled cytochemically stained ER in CHO-K1 cells in electron tomograms from different cell depths. The obtained stuctures demonstrate in 3D that the sheets are concentrated in the upper regions near the nucleus, whereas long tubules are found in the peripheral parts - the thin lamellipodium - and towards the bottom of the cell near substrate (I: Fig. 2, 6A and B).

The visualization of the ER with the same methods in NRK-52E and especially Huh-7 cells revealed, however, a different pattern. In NRK-52E cells, the sheet-rich region around the nucleus was wide and some sheets extended even to the periphery, where we observed a small network of tubules (II: Fig. 2D). The ER in Huh-7 cells appeared even more enriched in sheets and they often filled the entire lamellipodium (II: Fig. 2B and C). However, this pattern had some variability among the cells, such that cells with peripheral tubular networks were not difficult to find.

1.1.2 Endoplasmic reticulum sheets can be fenestrated (I; II)

A detailed inspection of sheet structures at EM level brought up an additional factor of variation between cells. The sheets in Huh-7 cells had numerous fenestrations (unpublished Fig. 5; II: Fig. 3). The sheets in CHO-K1, HeLa, Vero and NRK-52E cells, on the other hand, had fenestrations only occasionally and were for the most part intact (I: Fig. 1B, 2, 6A and B; II: Fig. 3B, E, F and 4B) . The fenestrations were roundish with a size beyond the diffraction limited LM, which is not able to resolve objects that are closer to one another than about 200 nm. Mean diameter of the fenestrations was 75 nm, although the size and shape were not completely uniform in agreement with previous results (Lieberman, 1970; Tani *et al.*, 1975; Brown, 1978). We observed microtubules passing through fenestrations as shown in a tomographic model derived from a juxta-nuclear region of a Huh-7 cell (II: Fig. 4). Stabilization of the fenestrations did not, however, involve cytoskeleton. Treatment of Huh-7 cells with nocodazole or latrunculin A which depolymerize microtubules or actin filaments, respectively, did not abolish fenestrations (unpublished Fig. 5A and B, see also CLEM in Fig. 7). Furthermore, the effect of several fixation methods on fenestrations was evaluated. Samples were prepared with high pressure freezing and freeze substitution (HPF/FS, II: Fig. 4), with glutaraldehyde and a mixture of glutaraldehyde and paraformaldehyde (unpublished Fig. 5C-F). We additionally tested the effect of several treatments or compounds that could potentially affect ER morphology, such as expression of recombinant proteins (Snapp *et al.*, 2003), cytochemical staining and buffering agent (Griffiths, 1993). The result was that we could not observe differences in appearance or quantity of the fenestrations after any treatment or sample preparation procedure (unpublished Fig. 5; II: Fig. 3 and 4).

The quantity of sheet fenestrations in Huh-7 cells relative to NRK-52E cells was confirmed by measuring the length distributions of ER profiles. The fenestrations are expected to increase the proportion of short profiles and decrease long profiles, since the transverse sections of fenestrated sheets have gaps as opposed to long profiles of intact sheets (unpublished Fig. 5; II: Fig. 3 and 4). Indeed, the interphase Huh-7 cells had significantly more short and less long profiles than the interphase NRK-52E cells ($p<0.05$, II: Fig. 7).

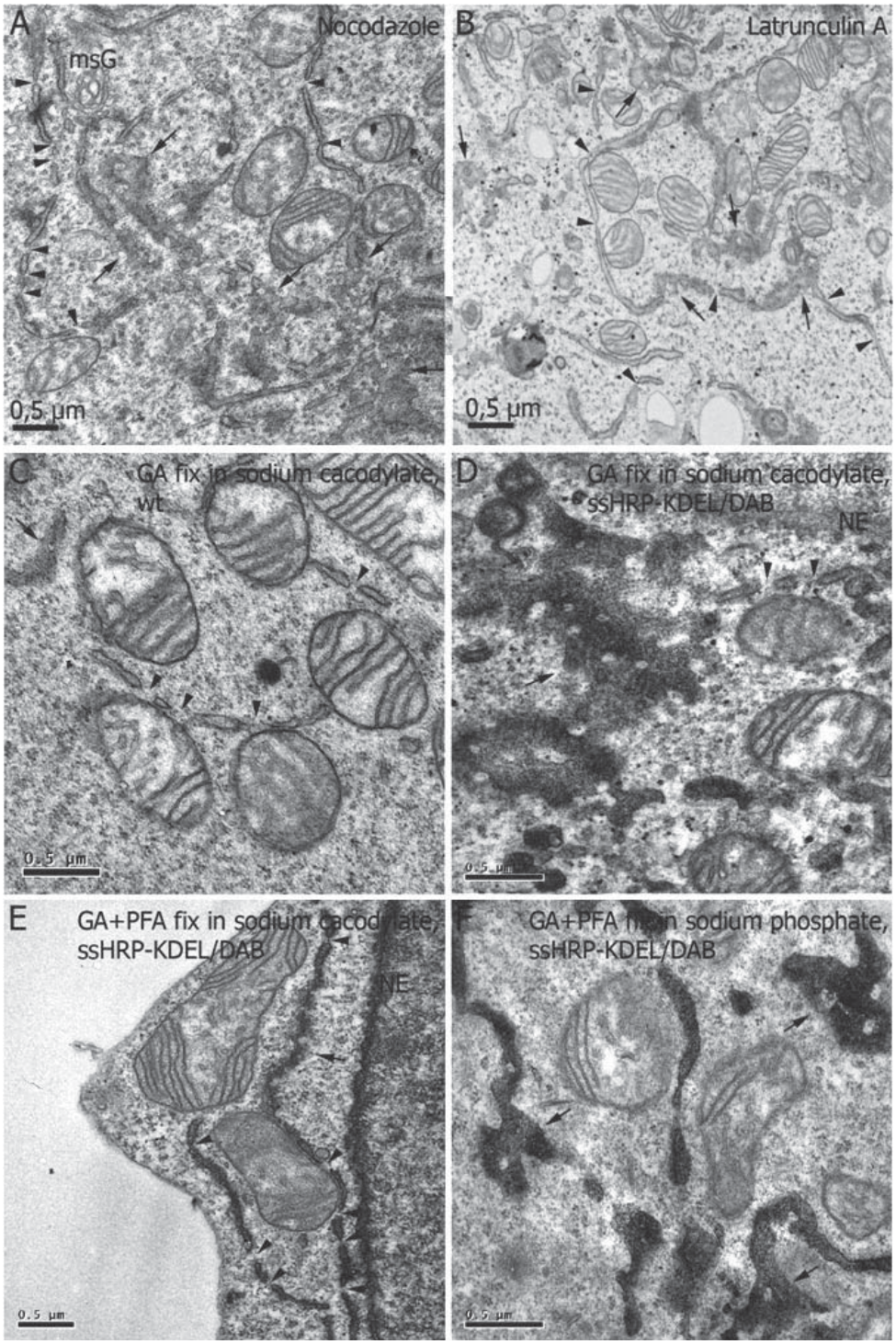
A
Nocodazole
msG
0,5 µm
B
Latrunculin A
0,5 µm
C
GA fix in sodium cacodylate,
wt
0,5 µm
D
GA fix in sodium cacodylate,
ssHRP-KDEL/DAB
NE
0,5 µm
E
GA+PFA fix in sodium cacodylate,
ssHRP-KDEL/DAB
NE
0,5 µm
F
GA+PFA fix in sodium phosphate,
ssHRP-KDEL/DAB
0,5 µm

Figure 5. The fenestrations of ER sheets in Huh-7 cells are not abolished by depolymerization of the cytoskeleton, cytochemical staining, use of different fixatives or buffering agents. Wt Huh-7 cells were treated with nocodazole (A) or latrunculin A (B) to depolymerize microtubules and actin filaments, respectively. The cells show multiple gaps (arrow heads) in transversely sectioned sheets and fenestrations in longitudinally sectioned sheets (arrows), indicating retention of the usual fenestrated sheet morphology in these cells. The same morphological features were observed after fixation with glutaraldehyde (C, D) or a combination of glutaraldehyde and paraformaldehyde (E and F), in wt (C) or cytochemically stained (D-F) cells or buffering the fixative solution with sodium cacodylate (C-E) or sodium phosphate (F).

1.1.3 Remodelling and mobility of sheets: fenestrated sheets of Huh-7 undergo less fusion and fission than intact sheets of NRK-52E (I; II)

Earlier studies show that ER networking is managed by branching of tubules and sheets. Our ET analysis of interphase ER in CHO-K1 cells revealed three types of branch points, between two tubules, a tubule and a sheet and two sheets (I: Fig. 2C). The dynamics of these structures has been only partially characterized and completely concentrated on tubular dynamics (Lee and Chen, 1988; Waterman-Storer and Salmon, 1998; Wozniak *et al.*, 2009). We were challenged by the lack of knowledge on sheet dynamics and decided to compare the movement of intact sheets in NRK-52E to fenestrated sheets in Huh-7. The advantage of these cell types is that both have sheets in the thin lamellipodial region with a single layer of ER. Thus, the sheets can be clearly resolved with a LM and they move mostly only in lateral direction because of the restricted space. First, we observed four characteristic events in sheet dynamics: 1) remains as a sheet, 2) fuses to other sheet, 3) transforms into tubules or 4) splits into two sheets, and scored their occurrence during 60 s (1 frame/s) videos (II: Fig. 10). It appeared that the fenestrated sheets were much more persistent than the intact sheets in NRK-52E, which were constantly splitting and fusing with other sheets. Second, we developed a novel method to measure lateral movement of sheets by calculating the center point of the sheet mass and following its movement over time (II: materials and methods). However, the mean mobilities of the two sheet types were almost identical, roughly 100 nm/s and the movements concentrated around one region (II: Supplemental Fig. 1 and 2). This does not mean that the sheets were immobilized to one place or moved all the time at a regular pace, since occasionally they showed faster and longer movements.

1.2 Homotypic fusion by p97/p37 maintains Golgi and endoplasmic reticulum architecture during interphase (III)

ER and Golgi membranes undergo a constant rearrangement that requires homotypic fusion of the membranes. Previous studies have implicated the ATPase p97 and its cofactor p47 in the processes of Golgi, ER and NE reassembly (Uchiyama *et al.*, 2002; 2003; Hetzer *et al.*, 2001). However, during interphase p47 is found within the nucleus. To elucidate whether p97 has another cofactor mediating organelle fusion during interphase, a clone similar in sequence to p47 and capable of binding p97 was identified with a yeast-two hybrid screen of a brain cDNA library that is rich in cDNAs from non-proliferating cells (III: Fig. 1). The clone was called p37 according to its predicted molecular weight and was found to be expressed in several other tissues than brain. Unlike p47, it did not contain nuclear localization signals or a defined ubiquitin binding domain and was shown to form a complex with p97 in an ubiquitin independent manner (III: Fig. 2). Binding of p97 to p47, Ufd1/Npl4 or VCP/p97-interacting protein inhibited the binding of p37 to p97 and thus, was mutually exclusive.

The localization of p97/p37 within interphase cells was determined by indirect immunofluorescence. Colocalization of p37 with a *cis*-Golgi marker, GM130 (Nakamura *et al.*, 1995), and an ER marker, protein disulfide isomerase, indicated that p37 is found in both organelles (III: Fig. 3). Next, the effect of blocking the action of p37 either by microinjecting p37-antibodies or knocking down p37 with siRNA in NRK or Hela cells, respectively, was studied. The microinjected cells were incubated for 4h during which all cells entering mitosis were removed, while the siRNA treatment lasted for 48 h. The result in these two treatment groups was nevertheless the same: While the Golgi stayed close to the nucleus, quantification in thin section electron micrographs revealed an increase in Golgi tubules and vesicles and reduced cisternal membranes as compared with control cells (III: Fig. 4A-C).

The role of p37 in the maintenance of ER network was studied in CHO-K1/Hsp47-GFP cells similarly, except the structures were only quantified from confocal microscope images of live cells. The ER had a larger mesh size after the treatments and the number of three-way junctions was reduced by over 50% (III: Fig. 5). In addition, we observed ER around the Golgi area in thin EM sections of anti-p37 microinjected NRK cells, but here we did not

detect obvious changes as compared with control cells. These results indicate that p97/p37 is involved in the maintenance of interphase Golgi and ER structures, but ER maintenance might have some variability between cell types.

2. Towards fragmentation – the endoplasmic reticulum and Golgi are reorganized during mitosis

2.1 Fenestration, tubulation and alignment of endoplasmic reticulum membranes varies during mitosis

2.1.1 Mitotic cells show an increased number of short endoplasmic reticulum profiles, but their alignment varies between cells (I; II)

Disassembly of mammalian Golgi apparatus during mitosis is well documented and involves shedding of vesicles, tubulation and fenestration of cisternal parts until the partitioning units, the mitotic Golgi clusters composed of vesicles and tubules, are formed (Lucocq *et al*., 1987; Shima *et al.*, 1998; Jokitalo *et al.*, 2001). Inheritance of ER membranes, on the other hand, has been studied relatively little and systematic studies combining LM, EM and quantification are missing. Therefore, we started our studies of mitotic ER organization in mammalian cells using the combined approach and CHO-K1 cells (I). There, visual evaluation of the ER in confocal optical sections (I: Fig. 3) revealed that the ER is a dense network during mitosis, whereas thin EM sections (I: Fig. 4 and 7) showed numerous short ER profiles. Quantification of ER profile lengths and branch points from images obtained with both techniques confirmed the impression: the number of short ER profiles and branch points increased during mitosis (I: Fig. 5, Table 1 and 2).

A similar tendency for short profiles was observed also in HeLa, Vero, Huh-7 and NRK-52E cells during mitosis (II: Fig. 5 and 6). The length distribution of ER profiles was quantified in mitotic Huh-7 and NRK-52E cells, which demonstrated that both cell types gained short and lost long profiles as compared to interphase (II: Fig. 7).

Despite these similarities, the spatial organization of ER membranes seemed to vary between different cell types. When we scrutinized late prometaphase to anaphase CHO-K1

cells, ER profiles were quite homogenously distributed outside the mitotic spindle and their alignment in any special direction was minimal (I: Fig. 3B-F, 4B and C). In contrast, in other cell types ER profiles often, although not exclusively, aligned along the plasma membrane forming long lines (II: Fig. 5 and 6). In EM images, the long lines were resolved to consist of several profiles separated from each other by small gaps and constriction sites. Sometimes there were multiple concentric layers of lines (II: Fig. 6C1-2). These features were evident in live cell LM as well as in chemically fixed or HPF/FS EM samples using either of our GFP or horse radish peroxidase (HRP) -coupled ER markers or wt cells. However, we found considerable variation in the alignment of the network in wide-field LM images or thin EM sections even within a single cell line, as quantified for Huh-7 cells (II: Fig. 5B and C).

2.1.2 The endoplasmic reticulum in mitotic CHO-K1 cells is predominantly composed of tubules while Huh-7, NRK-52E, Vero and Hela cells have fenestrated sheets and tubules (I; II)

An additional thin section ER profile type that we frequently observed in mitotic cells was the longitudinal section of fenestrated ER sheet. It was missing in CHO-K1 cells, at least during the mid-phases of mitosis, and could only be resolved by EM in Huh-7, NRK-52E, HeLa and Vero cells (II: Fig. 5D-F and 6D1-3). To fully understand the three-dimensional configuration of the ER in mitotic cells, we subjected the CHO-K1, Huh-7 and NRK-52E cells to ET. The analysis revealed that the ER was primarily tubular in CHO-K1 cells (see models in I: Fig. 6C and II: Fig. 1 that are from chemically fixed and HPF/FS material, respectively) and composed of fenestrated sheets in addition to tubules in NRK-52E cells (ER model in II: Fig. 6E of HPF/FS material) or Huh-7 cells (CLEM sample in II: Video 5) during metaphase. Furthermore, it directly demonstrated that the gapped long lines of ER profiles in thin EM sections are derived from fenestrated sheets. However, sometimes the sheets were so extensively fenestrated that it was hard to distinguish fenestrated sheets from tight tubular networks.

2.2 The nuclear envelope is transformed into a part of endoplasmic reticulum network during mitosis (I; II)

The largest ER sheet of the mammalian cell, the NE, breaks down during prophase to prometaphase. LM analysis has revealed that the NE components, such as the LBR, diffuse into peripheral ER, which remains continuous, and concentrate in the reforming NE after chromosome separation (Ellenberg *et al.*, 1997; Yang *et al.*, 1997). We, too, observed this phenomenon (I: supplemental video 4). In order to follow the fate of the NE at EM level, we observed CHO-K1 cells expressing LBR tagged with HRP. Analysis of cytochemically stained ER profiles in thin sections confirmed the findings done at LM level: LBR began to distribute to peripheral ER during prophase and this was accelerated after NEBD (I: Fig. 7). The NEBD proceeded through perforation of the NE, which was seen as wide gaps in NE profiles. Nevertheless, the remaining NE profiles kept their darkly stained, thin appearance and nuclear pores until late prometaphase. At metaphase, the NE could no longer be discerned from the rest of the ER: LBR labeled structures looked identical to the ER in CHO-K1 cells expressing a general ER marker ssHRP-KDEL (I: Fig. 7F and 4B). However, one difference between the two labels was that with LBR-HRP we observed more often unstained ER profiles, which could be directly continuous with the stained profiles. After NEBD and until late anaphase, the ER was essentially excluded from the region containing the spindle and chromosomes. In late anaphase, short and stained ER profiles started to gather around the chromosomes, but their thinning occurred only after somewhat longer stretches were formed.

An ET study of the same cells confirmed the findings above. In addition, it directly showed that LBR redistributed to peripheral ER network that was mainly tubular already in prometaphase and kept this organization until telophase (I: Fig. 8). The disassembling and reforming NE was visualized as small sheets (shown in red in the model of a telophase cell, I: Fig. 8C). In all of our ER models, the ER including the NE is a continuous, non-fragmented, structure throughout the cell cycle (I, II).

2.3 Membrane fusion by p97/p37 recreates endoplasmic reticulum and Golgi architectures after mitosis (III; unpublished)

The division of the fusion activity between the different p97 cofactors seems clear: p47 participates in Golgi and ER reassembly after mitosis (Uchiyama *et al.*, 2002; 2003; Hetzer *et al.*, 2001) and p37 in the maintenance of the respective interphase structures. Most fusion activities seem to be inhibited during mitosis. For the p97/p47 complex, the inhibition is mediated by dissociation of p97 from the Golgi and phosphorylation of p47 (Uchiyama *et al.*, 2003). How is the fusion activity of p37 regulated during mitosis or when does it resume after mitosis? To study this question, NRK cells were microinjected at prophase or early prometaphase with anti-p37 antibodies and fixed after mitotic exit. Quantification of Golgi structures in these cells showed a similar but milder effect as interphase cells treated the same way: the proportion of cisternae was reduced and tubules increased significantly, while the number of vesicles was only somewhat higher than in control cells (unpublished Fig. 6; III: Fig. 4E).

To study the mild *in vivo* effect further, we measured cisternal lengths along their midlines in reassembled Golgi stacks (unpublished data). The collected data was significantly different in treated vs. control cells ($p<0.001$). It appeared that the longest cisternae were missing in anti-p37 injected cells as shown in the frequency distribution of different length categories (unpublished data, Table 1). In control cells, we measured cisternae that were up to 3.09 µm long, whereas the longest cisterna in anti-p37 injected cells was 1.29 µm. The proportion of >0.72 µm cisternae was 2.4—3.4 fold in control cells: 19% in quenched anti-p37 (n=606 cisternae in 10 cells) and 26% in random IgG samples (n=632 cisternae in 11 cells), as compared to 8% in anti-p37 injected cells (n=663 cisternae in 10 cells). In other words, this means that >0.72 µm cisternae constituted 40—51% of the total cisternal length in control cells, whereas this proportion was only 18% in anti-p37 injected cells.

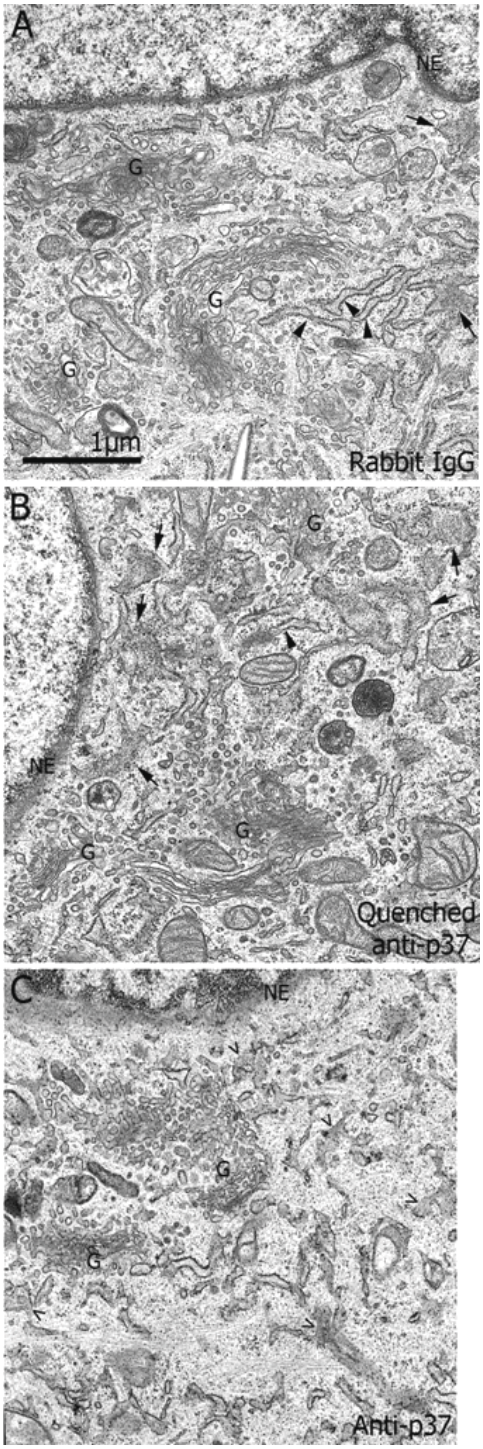

Figure 6. Morphology of the Golgi and ER in post-mitotic NRK cells after microinjection of anti-p37 or control antibodies into prophase cells. The Golgi (G) shows longer cisterna and less short profiles in control cells injected with rabbit IgG (A) or quenched anti-p37 (B) than in cells injected with anti-p37 (C). Similarly, the ER surrounding the Golgi in control cells shows long transverse sections (arrow heads) and large longitudinal sections (arrows) of intact sheets. In contrast, ER profiles are generally short and longitudinal sections of sheets small or fenestrated (open arrow heads) in anti-p37 microinjected cells. NE, nuclear envelope. The scale bar applies to all images.

Table 1. Frequency of cisternal lengths in different length categories in post-mitotic NRK cells after microinjection of anti-p37 or control antibodies into prophase cells. Cisternal lengths were measured along the midline of the cisternal profile in thin EM sections. The proportion of cisternae in different length catergories was determined after injection of p37-antibody, quenched p37-antibody or random IgG.

Cisternal length (µm)	% Membrane distribution		
	Anti-p37	Random IgG	Quenched anti-p37
0.000-0.199	7.3	8.5	8.0
0.120-0.239	23.2	23.7	12.4
0.240-0.359	24.6	18.4	17.9
0.360-0.479	16.6	10.9	14.7
0.480-0.599	12.9	10.4	11.2
0.600-0.719	7.7	9.6	9.4
0.720-0.839	2.9	6.2	8.5
0.840-0.959	2.7	3.9	6.5
0.960-1.079	1.5	3.3	2.3
1.080-1.199	0.3	2.2	2.9
1.200-1.319	0.3	1.7	2.1
1.320-	0.0	1.2	3.9

Direct demonstration of membrane fusion activity of the p97/p37 complex was obtained with *in vitro* Golgi reassembly assays (Rabouille *et al.*, 1995). The cisternal regrowth occurring from isolated mitotic Golgi membranes was shown to require the tethering system p115-GM130, cofactor VCIP135, but not its deubiquitinating activity, and a Golgi SNARE GS15 (III: Fig. 7 and 8).

The experiment of microinjecting anti-p37 in early mitosis was repeated in CHO-K1 cells to study ER reassembly. The CHO-K1/Hsp47-GFP cells were imaged by confocal live cell microscopy and quantification indicated again a milder effect than during interphase: the number of three-way junctions was decreased by approximately 40% (III: Fig. 5D). In thin EM sections of microinjected NRK cells, on the other hand, we observed small or perforated sheets and a high number of short ER profiles around the Golgi (unpublished data, Fig. 6).

The control cells had much more and larger intact sheets and less short ER profiles in the same area. Together these results suggest that the p97/p37 complex participates in the reassembly of the Golgi and ER structures after mitosis.

3. Ribosomes avoid high-curvature endoplasmic reticulum membranes

3.1 Ribosomal density on endoplasmic reticulum membranes decreases during mitosis in a curvature dependent manner (I; II)

Secretion, translation and vesicle transport are largely inhibited during mitosis (Warren, 1993; Le Breton *et al.*, 2005). Thus, concomitant with cell division and the change in ER morphology, we observed another change on ER membranes: a large number of ER-bound ribosomes were lost. The magnitude of the reduction was quantified in chemically fixed CHO-K1 (I), Huh-7 and NRK-52E cells (II), resulting in 70%, 45% and 43% reductions, respectively, compared to interphase situation. Since the density of ER-bound ribosomes differed between cell lines already during interphase being smallest in CHO-K1 (12 ribosomes/μm) and highest in NRK-52E cells (17 ribosomes/μm), the density in mitotic CHO-K1 cells was less than half (4 ribosomes/μm) of that in Huh-7 (8 ribosomes/μm) and NRK-52E cells (10 ribosomes/μm). Interestingly, the mitotic ER structures of these cells correlate perfectly with the observed numbers: the ER in CHO-K1 is mainly tubular, whereas Huh-7 and NRK-52E have fenestrated sheets and tubules. From these, tubular membranes have highest proportion of curved membranes, next highest is found in fenestrated sheets and then in flat sheets. Thus, the higher the membrane curvature, the less we found ER-bound ribosomes.

3.2 Structure-specific quantification reveals low density of ribosomes in many different types of highly curved endoplasmic reticulum membranes (II)

A mean density of ER-bound ribosomes derived from all ER membranes does not give straight answer to whether it is the membrane curvature that decreases the number of ribosomes unless we find cells with purely tubular or sheet-like ER. In that case there could still be doubt that some other mechanism leads to smooth tubules and rough sheets. We

therefore tackled the question by quantifying ribosomes separately on different types of ER membrane structures (II: Fig. 8): sheets, fenestrations and tubules. The structures were identified on thin EM sections of HPF/FS material based on the presence of ribosomes on membranes and knowledge of the usual structural dimensions derived from electron tomography (II: see materials and methods). Sheet edges were incorporated indirectly in the quantification, because ER-bound ribosomes on sheets were assessed both from transverse sections that mainly consist of flat membranes and from longitudinal sections, which more likely expose sheet edges.

The data obtained showed that all three high curvature membrane classes, tubules, sheet edges and fenestrations, contain lower density of ribosomes than flat sheet membranes (II: Table 1). Tubules had a lower density of ribosomes than fenestrations in sheets. This tendency was further confirmed by quantifying ribosomes on sheets and tubules in HPF/FS tomograms of Huh-7 and NRK-52E cells (II: Fig. 4).

3.3 High ribosomal concentration may stabilize sheets (I)

The lack of ribosomes on high-curvature membranes might be driven either by passive segregation of large polysomal complexes to flat membranes with a spacious lumen or ribosomes could actively stabilize sheets. Our results showing that reduction in ER-bound ribosomes correlates with increases in membrane curvature have two problems: 1) during interphase active protein synthesis could produce many different segregation systems and 2) during mitosis translation might be inhibited to varying degrees in different cell types. Thus, some cells might retain more ribosomes and synthesize more of an unknown sheet-stabilizing structural protein unrelated to ribosomal complexes during mitosis.

To analyze the role of ribosomes in stabilization of sheet structures, we treated interphase CHO-K1 cells with two drugs that interfere with translation in different ways. Puromycin causes premature chain termination, after which the smaller ribosomal subunit is quickly released and polysomal structure disrupted (Seiser and Nicchita, 2000). Cycloheximide, in contrast, blocks the elongation stage and stabilizes association of ribosomes with translocons (Seiser and Nicchita, 2000; Roy and Wonderlin, 2003). The treatments of 15 min had a clearly different effect on ER sheets. The ER was stripped of ribosomes with

puromycin (45% reduction) and was composed of tubules and extensively perforated sheets as shown by thin section EM and electron tomography (I: Fig. 9E and 10B). ER in the corresponding cycloheximide treated cells did not loose ribosomes (9% decrease) or change ER structure (I: Fig. 9C and 10A). A longer puromycin treatment produced similar results than the short one. A longer cycloheximide treatment, on the other hand, resulted in partial stripping of ribosomes (32% reduction) and morphology with tubules and perforated sheets. We confirmed the results by quantifying small ribosomal subunit S6-immunofluorescence staining in semi-permeabilized, salt-washed CHO-K1 cells (I: Fig. 9C). Thus, a mere translational block is not enough to transform sheets to tubules, but seems to require the dissociation of ribosomes. It appears that a high concentration of ribosomal assemblies may actively stabilize sheets.

4. Combining light and electron microscopy to study the effects of microtubule depolymerization on endoplasmic reticulum and Golgi structure

4.1 Comparing light and electron microscopy images of the Golgi (unpublished)

The possibility to combine LM with EM has proven to be important in our studies of ER and Golgi morphogenesis. Dynamic events, the overall pattern and total quantities are best appreciated with LM that allows also screening of relatively large sample sizes. Other benefits include easy interpretation of the results, since only the labeled protein is visible, quick sample preparation and good availability of the microscopes. EM, on the other hand, provides superior resolution and reveals not only the organelle or molecule of interest but other cellular structures surrounding it.

The importance of good resolution on structure identification is exemplified well by analysis of Golgi and ER, since they have complicated structures, in which many dimensions are beyond the resolution power of the conventional diffraction limited LM. Here, localization and structure of Golgi membranes in NRK-52E cells was analysed with the help of immunofluorescence staining of endogenous *cis*-Golgi matrix protein, GM130 for LM (Nakamura *et al.*, 1995) and in cytochemically stained cells stably expressing the *trans*-Golgi marker α2,6-sialyltransferase coupled to HRP (ST-HRP, Connolly *et al.*, 1994;

Stinchcombe *et al.*, 1995; Jokitalo *et al.*, 2001; unpublished Fig. 7). During interphase, the Golgi appears as a perinuclear ribbon in LM (Fig. 7A). In EM, the ribbon is resolved further (unpublished Fig. 6A) to consist of cisternal stacks connected to each other by tubular structures (Farquhar, 1981; Rambourg, 1987). During mitosis, the structure of the Golgi changes dramatically. At LM level, it appears as dispersed bright spots or a combination of spots and weak haze (Fig. 7A, a telophase NRK-52E cell) depending on the detection system and cells used (Zaal, 1999; Shima *et al*., 1998; Axelsson and Warren, 2004). The structural identity of the spots and haze is revealed by EM showing that they are composed of vesicles or clusters of vesicles and tubules (Lucocq *et al*., 1987; Jokitalo, 2001). Figure 7B depicts a late anaphase/early telophase NRK-52E cell.

Morphology and localization of the Golgi has been shown to change after depolymerization of microtubules (Rogalski *et al.*, 1984; Cole *et al.*, 1996; Hammond and Glick, 2000). LM demonstrates that the interphase Golgi ribbon scatters into dispersed punctae of variable size after nocodazole treatment (Fig. 7C). Coupling to EM imaging is required to find out that their ultrastructure is not the same as during mitosis but rather ministacks of Golgi cisternae (Fig. 7D; Cole *et al.*, 1996).

4.2 Correlative light electron microscopy of the endoplasmic reticulum (unpublished)

The combination of LM with EM in its purest form is correlative light electron microscopy (CLEM), which is used to study the same cells or structures first in LM and then in EM. We used CLEM to study the consequences of microtubule depolymerization on ER dynamics and morphology in Huh-7 cells cotransfected with ssGFP-KDEL and ssHRP-KDEL as ER markers for LM and EM, respectively (see materials and methods in II). Microtubule depolymerization has been shown to lead to clustering and codistribution of ER exit sites with IC or ministacks of Golgi (Cole *et al.*, 1996; Hammond and Glick, 2000), slow retraction of the ER (Terasaki *et al.*, 1986) or collapse into cisternae (Lu *et al.*, 2009) or cisternae-like structures (Shibata *et al.*, 2008) and cessation of ER movements (Waterman-Storer and Salmon, 1998, Wozniak *et al.*, 2009).

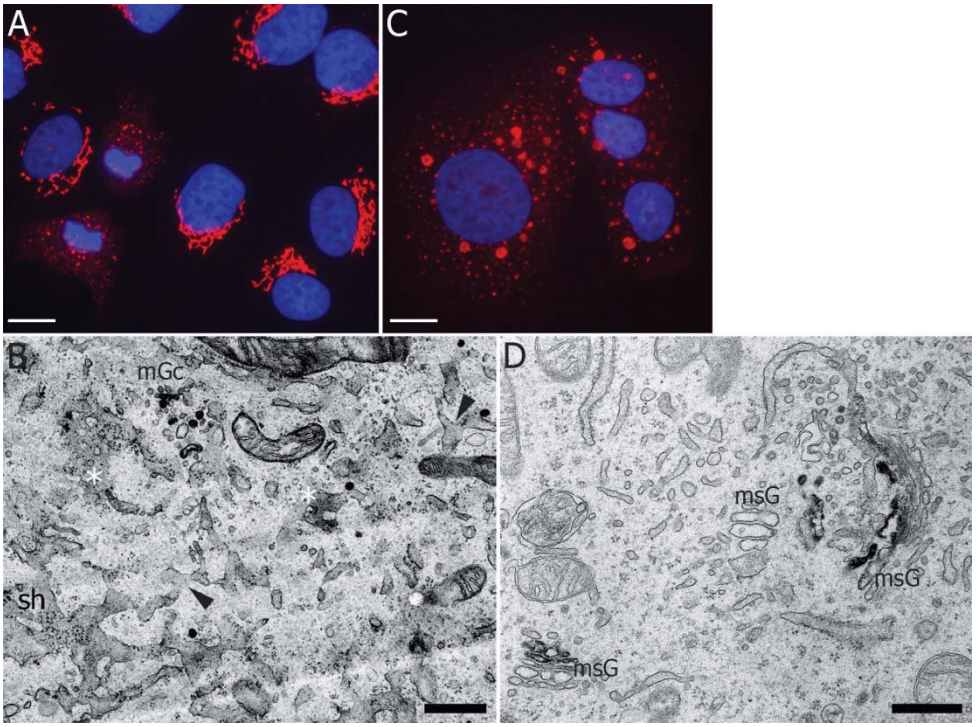

Figure 7. The visualization of structural alterations of the Golgi apparatus in NRK-52E cells by comparative LM and EM. The cells expressing ST-HRP were (A-C) stained with GM130 antibodies for fluorescence LM or (B and D) cytochemically stained for EM. (A)The Golgi appears as a typical perinuclear ribbon in interphase cells and as spots and weak haze in the early telophase cells. (B) At ultrastructural level, the mitotic spots are resolved to consist of mitotic Golgi clusters (mGc) of vesicles and tubules. Image B is derived from a late anaphase/early telophase cell and shows also fenestrated ER sheets (asterisks) and branch points (arrow heads) typical for a dividing cell. (C) When the microtubules of interphase cells are depolymerised with nocodazole, scattered punctae of variable size are seen with LM. (D) Again, by EM the structure of the punctae is revealed to be, not clusters of vesicles and tubules, but ministacks of Golgi cisternae (msG). Bars are 10 μm in LM images and 0.5 μm in EM images.

We too imaged the cells every 3s with a wide-field microscope for 90 s and observed that ER, including both sheets and tubules, moved continuously before (unpublished data, Fig. 8A) and slowed down during (Fig. 8B) nocodazole treatment. The peripheral ER normally composed of sheets and tubules (Fig. 8A and C) in these cells was converted into a homogenous mass (Fig. 8B and D). In addition, dispersed fluorescent spots - that probably correspond to ER exit sites or IC because of the KDEL-tag in the marker protein - multiplied and grew in size during the treatment. The cell, which was immediately processed for

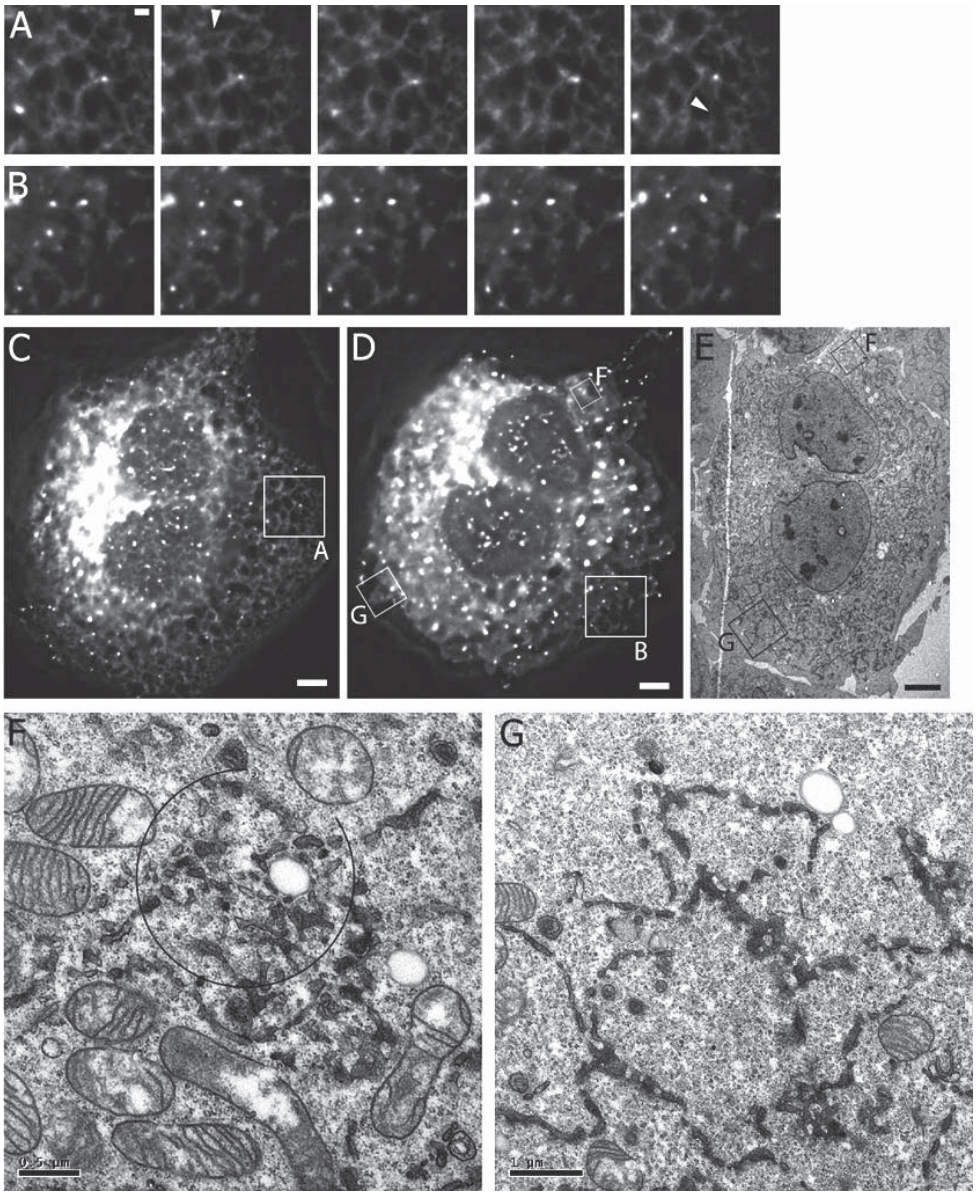

Figure 8. CLEM of a Huh-7 cell during nocodazole treatment. A Huh-7 cell coexpressing ER markers ssGFP-KDEL and ssHRP-KDEL was imaged with a wide-field microscope every 3 s before (A) and during (B) nocodazole treatment. Panel A depicts normal remodelling of ER network in consequtive frames (arrow heads point to changing regions). (B, D) In the presence of nocodazole, the network does not move and changes into a homogenous mass with an increased number of large bright punctae. (E) After acquisition of the last image, the cell was chemically fixed, cytochemically stained and processed for EM. F and G show boxed areas in E and D in high magnification. The ER contains compact tubular regions, here around a lipid body (circled area in F), in addition to abundant fenestrated sheets (G). Scale bar is 1 µm in LM panel A, which applies also to panel B, 5 µm in C-E, 0,5 µm in F and 1 µm in G.

EM after acquiring the last image, had abundant sheet profiles in thin section images (Fig. 8E and G). The sheets had gapped transverse sections arranged in long lines and perforations in longitudinal sections typical for the fenestrated sheets in Huh-7 cells. However, in addition to sheets, we observed compact regions with tubular profiles (Fig. 8F). The tubular profiles had few ribosomes, were short and not arranged in lines but rather had arbitrary orientations, which is typical for tubular profiles. These features were also observed in wt Huh-7 and in NRK-52E cells expressing the *trans*-Golgi marker, ST-HRP, treated with nocodazole and processed for EM (unpublished data, Fig. 9). The tubular regions were found quite often around lipid bodies or next to ministacks of Golgi and were, in turn, often surrounded by ER sheet profiles.

ER origin of these tubules was determined by cytochemical staining of ssHRP-KDEL and the presence of some ribosomes on the tubular membranes or membranes that were directly continuous with the tubular profiles. The cytochemically stained membrane profiles of ST-HRP expressing cells were clearly distinct from these tubules: they were found only in ministacks of Golgi. The ER tubules close to the ministacks of Golgi may correspond to the clustered ER exit sites (Hammond and Glick, 2000). However, partial contribution of the IC to the structures cannot be excluded. Thus, destruction of microtubules with nocodazole does not lead to conversion of all ER tubules to sheets, but compact tubular areas also contribute to the phenotype observed by LM.

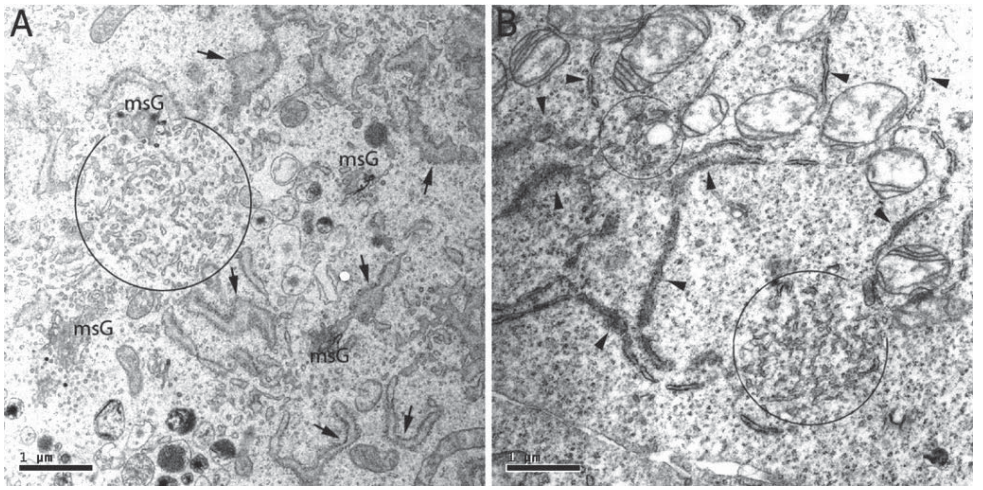

Figure 9. NRK-52E and Huh-7 cells treated with nocodazole show compact tubular areas in addition to abundant sheets. Nocodazole treated NRK-52E (A) and Huh-7 (B) cells have compact tubular areas of ER (circled) that are found often close to ministacks of Golgi (msG) or surrounding lipid droplets (smaller circle in B). In the surrounding ER, many large intact sheets (arrows in A) or fenestrated sheets (arrow heads in B) are seen. The NRK-52E cell expresses the trans-Golgi marker ST-HRP and has been cytochemically stained.

Discussion

Studies about interphase ER and Golgi architecture have revealed several mechanisms that account for their characteristic structure. Morphogenesis during and after mitosis, on the other hand, are less well established for the ER than Golgi. In addition to specific structural proteins, our observations together with previous work have led me consider the following factors as particularly important for ER and Golgi morphogenesis:

1) Protein synthesis and secretion
2) Membrane fusion and fission
3) Cytoskeleton

I will discuss these points in relation to our results, the relevant literature and other morphogenetic factors during the cell cycle with the emphasis on ER.

1. Polysomes and the effect of Ca^{2+} on the endoplasmic reticulum

Our experiments, where ER structures are related to the density of ER-bound ribosomes suggest that 1) ribosomes avoid high curvature membranes and 2) intact sheet morphology is stabilized directly or indirectly by a sufficient density of ribosomes (Fig. 10). The experiments with puromycin and cycloheximide in CHO-K1 cells (I) are especially important for the latter conclusion, the stabilization of sheets. The use of translation inhibiting drugs is not, however, problem free and can have many secondary effects. It has been shown that puromycin treatment induces Ca^{2+} leak-out from the ER via "empty" ribosomes on translocation channels (Roy and Wonderlin, 2003). ER structure seems to be somewhat controlled by cytosolic Ca^{2+}, since low concentrations are needed for ER network formation, whereas high concentrations might fragment the ER (Dreier and Rapoport, 2000; Voelz *et al*., 2006). In contrast to puromycin, cycloheximide treatment does not result in Ca^{2+} leakage or depletion of ER Ca^{2+} stores, since it stabilizes the interaction between ribosomes and translocation channels (Seiser and Nicchita, 2000; Roy and Wonderlin, 2003; Van

Coppenolle *et al*., 2004). This raises the possibility that there could be some stable Ca^{2+} responsive protein that regulates stabilization of sheets independent of ribosomes. If this was the case, we would still expect to see the same morphologies after the treatments with puromycin and cycloheximide (I): 15 min puromycin treatment that enables Ca^{2+} leakage would cause fenestration and tubulation of ER sheets - that could be seen alternatively as a sign of ER network formation or fragmentation - and cycloheximide treatment would not cause such a structural change. In other words, increased cytosolic Ca^{2+} concentration in puromycin treated cells would change the activity of the Ca^{2+} responsive protein producing the structural change of the ER. The amount of this stable protein would not yet be affected by the 15 min translational block and it would keep stabilizing sheets in cycloheximide treated cells, where Ca^{2+} leakage to cytosol does not happen. However, after 2 h translational block this protein could be already depleted and this would result in fenestration and tubulation of ER sheets as well. At present, we cannot exclude this possibility. Nevertheless, the exact role Ca^{2+} plays on changing ER structures is far from clear and is lacking EM level 3D characterization. In addition, in our quantifications the densities of ER-bound ribosomes correlate very well with the observed ER structures making it likely that ribosomes have a role in stabilization of sheets. Thus, Ca^{2+} may control some aspects of ER morphogenesis, such as branch point generation by p22 (Andrade *et al*., 2004), but this may not be mutually exclusive with our proposed mechanism that the integrity of the sheets is dependent on ribosomal density.

The progression of mitosis is dependent on Ca^{2+} signals (Ratan *et al*., 1988; Tombes and Borisy, 1989; Parry *et al*., 2005) and thus, Ca^{2+} might control the mitotic structural change of the ER. Could Ca^{2+} leakage through the translocon occur during mitosis, when translation is inhibited? The probable answer is that it might if the translocon channel leaks Ca^{2+} under normal physiological conditions, which is currently unclear (Roy and Wonderlin, 2003; Van Coppenolle *et al*., 2004; Ong *et al*., 2007; Amer *et al*., 2009). In Hela cells, however, mitotic polysomes are arrested in the elongation phase of translation (Sivan *et al*., 2007). Since cycloheximide arrests elongation as well and Ca^{2+} leakage does not occur during cycloheximide treatment (Van Coppenolle *et al*., 2004), it is likely that at least in Hela cells, mitotic Ca^{2+} signals are not moving extensively through the translocons.

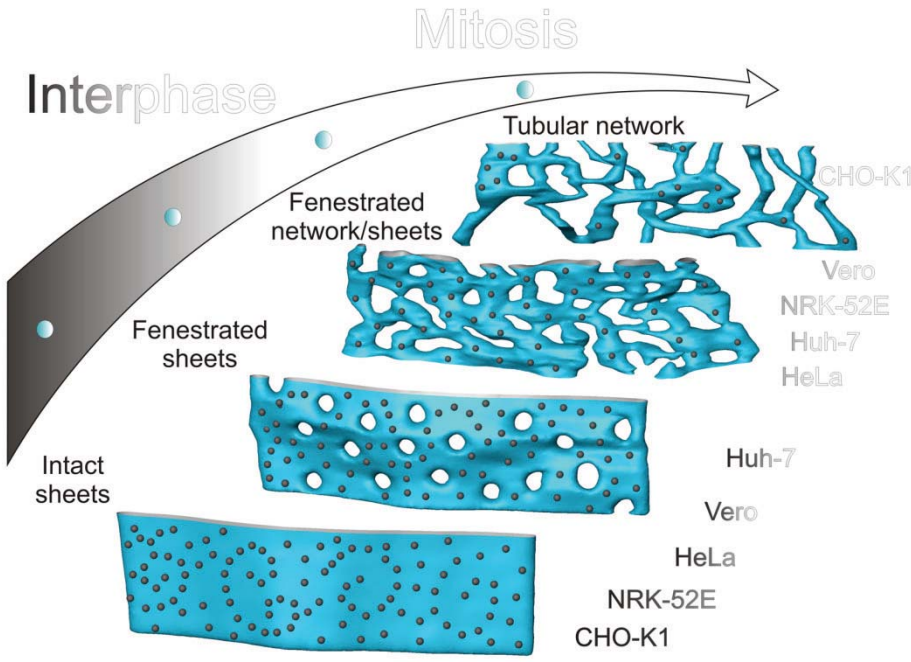

Figure 10. A model of mitotic sheet-to-tubule transformation. The transformation of RER starts from intact sheets or fenestrated sheets and proceeds towards a more fenestrated direction that can eventually produce structures resembling tubular networks. The starting and the end point vary between different cells, but the direction of the change is the same. The extent of the change correlates with the density of ER-bound ribosomes. The color gradients symbolize the progression from interphase to metaphase.

2. Protein synthesis and secretion

The Golgi and ER have been reported to expand together, when there is a demand for high rate of protein synthesis and secretion during interphase. Structurally this means that ribosome studded ER sheets proliferate and Golgi stacks and vesicles multiply, cisternae elongate or TGN expands towards the cell periphery (Wiest *et al*., 1990; Ueno *et al*., 2010a and b). This is supported by our results showing that intact ER sheet morphology is accompanied by high density of ribosomes (I; II).

Do Golgi and ER morphologies go together also during mitosis? Secretion is stopped and the Golgi disassembled in mammalian cells (Warren, 1993). Plant and yeast have dispersed Golgi stacks throughout the cell cycle, which could reflect the need to continuously secrete new cell wall material (Makarow, 1988). If only secretion dictated ER and Golgi structures, we should probably not observe variation in their mitotic morphologies between different mammalian cells. The truth is that there is variation in both Golgi and ER morphologies during mitosis (II; Maul and Brinkley, 1970; Moskalewski and Thyberg, 1990). In addition, in Hela cells, the extent of Golgi and ER disassembly appear uncoupled: the Golgi breaks down totally into vesicles and tubules (Lucocq *et al*., 1987), but the ER has a lot of fenestrated sheets (II; Mullins, 1984). In both organelles, the scale measuring the extent of dissassembly seems to be from retention of the cisternal parts, via production of fenestrated sheets to production of tubules and, for Golgi, vesicles.

While we do not know, why mitotic Golgi structures vary, our hypothesis to explain the different ER morphologies is that there are different amount of ribosomes – that may be stalled to elongation stage or active – on the ER of different cell types during mitosis. Retention of ribosomes on the ER and more cisternal morphology may allow quicker recovery of interphase activities and NE assembly after mitosis (Anderson and Hetzer, 2008). However, curvature of ER membranes during mitosis – tubulation and fenestration – might be needed for providing elastic support for the spindle (Liu and Zheng, 2009) or, perhaps, free passage of material in the cytoplasm (II; Novak *et al*., 2009). Furthermore, a study in yeast shows that tightly stacked ER sheets, karmellae, are not inherited normally but remain in the mother cell (Wright *et al*., 1988).

3. Fusion and fission

There are many studies about fusion and fission of vesicles to and from the ER and Golgi and about different fusion factors catalyzing homotypic fusion of ER and Golgi membranes. Fission together with inhibition of fusion is also known to mediate Golgi disassembly during prophase. For the ER, on the other hand, fission of structures other than vesicles has not been convincingly shown. In live cell videos of Huh-7 cells, the ER tubules do not undergo

fission events. For example, if an extending ER tubule fuses with another membrane, it does not reseparate and retract back (Merja Joensuu, personal communication of unpublished observations). Retraction can, however, take place before the tubule fuses with the target membrane.

Vesicles have been shown to bud from the edges of Golgi cisternae, which are often fenestrated (Staehelin *et al*., 1990; Ladinsky *et al*., 1994; Sesso *et al*., 1994; Misteli and Warren, 1995a; Staehelin and Kang, 2008). Since vesicle membranes are all curved, the pre-curved membranes of fenestrated cisternae might allow easier vesicle budding and fission. We did not investigate this possibility for the fenestrated ER sheets, but it is not very likely according to our results in Huh-7 cells for the following reasons. First, fenestrations in the sheets are not exclusively found in sheet rims (II). Second, all sheets in the cells seem to have fenestrations, which is not compatible with the known structure and more scattered and punctate localization of ER exit sites along the ER network (Bannykh *et al*., 1996; Zeuschner *et al*., 2006). Our initial idea about the function of fenestrated sheets was that they could enhance tubule biogenesis from the sheets by a similar curvature-employing fission, or alternatively, pulling, mechanism. However, investigation of sheet dynamics indicated that the fenestrated sheets in Huh-7 cells undergo less fusion and fission events than intact sheets in NRK-52E cells and both sheet types transform to tubules equally well (II). Therefore, the conclusion is that the function of fenestrae is not to aid tubulogenesis. In addition, fenestrated sheets are not transient intermediates in sheet-to-tubule transformation, which is supported by their abundance at any given moment.

Fenestrations could be formed by fission events, where the lumenal membrane monolayers of cisterna contact each other and fuse creating a hole, as has been presented to happen during disassembly of the Golgi (Warren *et al*., 1995). One hypothesis is that the luminal polypeptide folding/modification machineries that associate with polysomes and translocons during protein synthesis provide a scaffold that prevents membranes from touching each other and thus, fenestra formation during interphase. During mitosis, when translation is inhibited, this fission prevention mechanism would be downregulated and fenestrations formed. However, these mitotic fission events should be somehow tightly controlled, since a high level of membrane fission would produce ER fragments instead or in addition to the

continuous network detected by fluorescence recovery after photo bleaching (Ellenberg *et al*., 1997) and ET (I; II). Here, it is worth mentioning that studying the continuity of ER during mitosis really required the above mentioned methods, since LM may not resolve isolated structures of high density from a network and the thin EM section profiles of isolated ER tubules and vesicles resemble those of a tubular network or fenestrated sheets.

The membrane fusion systems of the Golgi and ER are inhibited during mitosis (Lowe *et al*., 2000; Uchiyama *et al*., 2003). The inhibition was recently extended to p37 that is phosphorylated at mitosis and cannot bind to the Golgi, which enables Golgi disassembly (Kaneko *et al.*, 2010). When p37-antibody was microinjected into prophase NRK cells and the cells were observed after mitotic exit, the Golgi had still mitotic features (III: Fig. 4E; unpublished data, Fig. 6). This is explained by partial inhibition of fusion activities that catalyze Golgi assembly: p37 is absent, while p47 and NSF are present (Rabouille *et al*., 1995; Uchiyama *et al*., 2003). Our additional data showed that particularly the longest cisternae of the Golgi were missing (unpublished data, Table 1). Therefore, the p97/p37 complex could have a role in the slow increase of cisternal length as the cells progress from telophase to early G1, a phenomenon that has been detected earlier (Souter *et al.*, 1993; Shima *et al.*, 1998).

The same mitotically microinjected NRK cells showed changes in ER structures around the Golgi. We observed many short ER profiles and small or perforated sheets at EM level (unpublished data, Fig. 6). On the other hand, the CHO-K1 cells that were microinjected with p37-antibodies similarly to NRK cells, showed less three-way-junctions of ER at LM level (III: Fig. 5D). Is there a correlation between these LM and EM data derived from different cell types? Because of anti-p37 microinjection, the ER, like the Golgi, may still have some mitotic characteristics - tubules and fenestrated sheets - in the partial absence of fusion activity that normally reshapes ER structures such as resolves the fenestrations after mitosis. Fenestrated sheets give, along with tubules, short profiles in thin EM sections. The large meshed ER visible in LM could reflect the importance of fusion activity for formation of branch points and the failure of the diffraction limited LM to resolve the fenestrations within sheets.

On the other hand, microinjection of p37-antibodies during interphase gave results that are more difficult to correlate: CHO-K1 cells showed less three-way-junctions at LM, but NRK cells did not have at least obvious shortening of ER profiles around the Golgi at EM. This might mean that there are some differences in ER fusion systems after mitotic exit and during later phases of interphase and perhaps between different cell types. It is for example not known how p97/p37 chooses its fusion targets or whether it fuses all or just some ER subdomains.

Similar to our observations of short ER profiles after depletion of functional p37 during mitosis, shortening of ER profiles has been reported after knockdown of the ER fusion factor atlastin in interphase drosophila neurons (Orso *et al.*, 2008). However, the short ER profiles were interpreted as ER fragmentation based on partial retention of fluorescence during continuous bleaching of some GFP-KDEL-positive regions. Similarly, glutamate stimulation in neuronal cells has been shown to inhibit partially the diffusion of KDEL containing protein within ER (Kucharz *et al.*, 2009). Here, the ER took a form of short profiles in lines and although it, too, could be interpreted as fragmentation of ER, the authors do not rule out the possibility that the profiles are interconnected in 3D. Indeed, our results showing that short ER profiles may be derived from tubules and lines of short profiles from fenestrated sheets, underline the importance of 3D investigation before making conclusions about ER fragmentation. In addition, the use of KDEL-containing ER marker inevitably includes some of the IC (Marie *et al.*, 2009) and cis-Golgi (Pelham, 1990; Connolly *et al.*, 1994) in the fluorescent pattern and could partially explain why diffusion was restricted in both cases.

Our own interpretation of ER morphology in several cell types (I-III) and ER quantification in CHO-K1 cells is also based on labeling the compartment with KDEL and RDEL-containing proteins. Therefore some of the IC or cis-Golgi structures may have been included in the quantifications and probably somewhat exaggerate the amounts of short ER profiles in CHO-K1 cells especially during mitosis (Fliegel *et al.*, 1990; Jokitalo *et al.*, 2001). However, we could detect similar increase in short profiles in Huh-7 and NRK-52E cell types during mitosis (II) without the labeling. Here, we relied on identification of ER profiles based on the

presence of ribosomes on these profiles, since these cells retain more ER-bound ribosomes during mitosis than CHO-K1 cells. Moreover, we have analysed the ER structures in 3D and in high resolution with and without the markers throughout the cell cycle in all of the above cell types, which supported our conclusions from the quantifications (I, II).

4. Cytoskeleton

Several studies indicate that microtubules have many specific linkage points to both ER tubules and sheets and an important role in spreading and extending ER network during interphase (Klopfenstein *et al*., 1998; Sanderson *et al*., 2006; Ogawa-Goto *et al*., 2007; Grigoriev *et al*., 2008; Connell *et al*., 2009; Smyth *et al*., 2009; Park *et al*., 2010). Furthermore, experiments with nocodazole and microtubule motor proteins suggest that ER tubule biogenesis is dependent on microtubules (Wozniak *et al*., 2009). Lu *et al*., (2009) even claim that ER structure after nocodazole treatment and during mitosis is almost exclusively cisternal, because ER looses contacts with the microtubules during both situations.

This argument is, however, opposed by many findings. First, ER tubules and microtubules are often coaligned, but their alignment is not perfect (Terasaki *et al*., 1986). Second, tubular ER networks can be built from Xenopus and rat hepatocyte microsomes *in vitro* without inclusion of cytosol (Dreier and Rapoport, 2000, Lavoie *et al.*, 1996). Third, reticulon and DP1/REEP proteins are required and sufficient for tubule formation and their overexpression opposes the effects of nocodazole on ER network: tubular regions persist in contrast to the sheet-like morphology seen without overexpression (Voelz *et al*., 2006; Shibata *et al.*, 2008; Hu *et al*., 2009; Park *et al*., 2010). Fourth, we observe tubular ER also in the peripheral cortical parts of the cell during mitosis, although microtubules are mostly concentrated to the spindle (I, II).

When we treated cells with nocodazole, we observed that ER contained both abundant sheets and some compact tubular areas after microtubule depolymerization, but not the long tubules usually seen at the lamellipodia (unpublished data, Fig. 8 and 9). The compact tubular areas were surrounded by sheets. Interestingly, this is exactly the ER organization

build *in vitro* from rat hepatocyte microsomes in the presence of $MgCl_2$, GTP and ATP but no cytosol (Lavoie *et al*., 1996). The occurrence of these tubular areas is further supported by data showing partial colocalization of the ER and p22, an ER branch promoting protein, in punctate structures after nocodazole treatment (Andrade *et al.*, 2004). Thus, our results indicate that microtubules may be used for extension of ER tubules *in vivo*, but not for creation of tubular shapes as such. This conclusion is in agreement with some of our preliminary data about mitotic Huh-7 cells obtained with a new technique called serial block face scanning electron microscope (SEM, 3View®, Gatan, Inc.). With this technique, the specimen block is cut and the block face imaged repeatedly inside the SEM allowing 3D reconstruction of the structures from the resulting image series. When we look at the modeled ER structures within a metaphase Huh-7 cell covering almost the whole cell (unpublished data, Fig. 11), we see fenestrated and layered sheets and short tubules at the cortical parts. However, long tubules extend from the cortex towards the middle of the cell. It appears, therefore, that ER tubules contacting microtubules (the spindle of the metaphase cell), are extended, whereas cortical ER tubules lack this association and are short. Nevertheless, this idea still lacks proof, since many proteins mediating ER-microtubule contacts during interphase loose the contacts during mitosis (Vedrenne *et al.*, 2005; Benyamini *et al.*, 2009; Smyth *et al.*, 2009).

Another role for ER-bound microtubules is that they physically limit the diffusional mobility of polysomes, which may serve to segregate RER and SER (Nikonov 2002; 2007; Nikonov and Kreibich, 2003). In addition, their binding to ER is mediated by two candidate sheet forming proteins, p180 and CLIMP-63 (Klopfenstein *et al*., 1998; Ogawa-Goto *et al*., 2007). So, it is an interesting question, how much of sheet stabilization is dependent on microtubules. If only microtubules stabilized ER sheets, they should be lost after depolymerization of microtubules. In addition, ER morphology should be similar after nocodazole treatment and during mitosis in the outer cortical parts of ER that are not in touch with microtubules. However, many studies including ours suggest that ER has abundant cisternae after depolymerization of microtubules (unpublished data, Fig. 8 and 9**;**

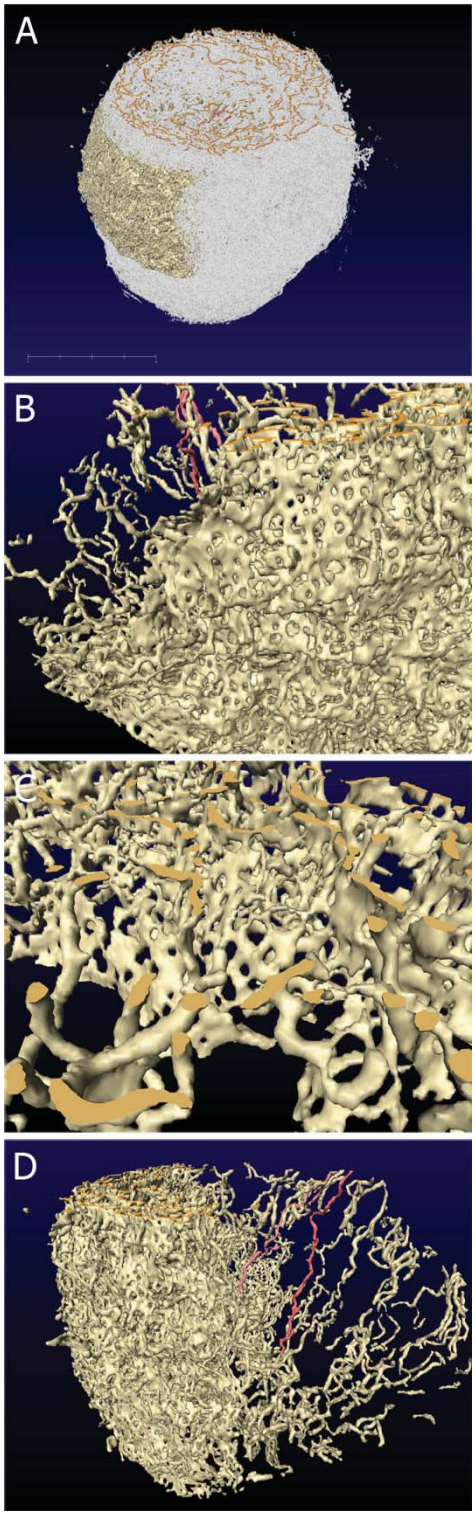

Figure 11. Long ER tubules of a metaphase Huh-7 cell are found in the spindle region, whereas cortical parts contain fenestrated sheets and short tubules. (A) A metaphase Huh-7 cell expessing ssHRP-KDEL was cytochemically stained and imaged with 3View SEM technique. The stained ER profiles were segmented automatically with the help of custom made functions in MatLab (unpublished data, Ilya Belevich). A smaller part of the model shown in yellow in A is depicted from different angles in B-D to allow better visualization of the structures. The ER profiles at the cutting surface are marked with orange and highlight ER layers in the cortex, whereas some of the longest tubular structures are shown in red and are not cortical (A-D). (B) Views of the outermost ER layer and (C) the inner ER layers show many fenestrated sheets and short tubules. (D) The long tubules that extend from the cortex towards the spindle are clearly seen in a side-view. The scale is 10 µm.

Terasaki *et al*., 1986; Shibata *et al*., 2008; Lu *et al*., 2009; Wozniak *et al*., 2009). Furthermore, our results show that mitotic sheets of the cortical ER are more fenestrated than sheets observed after nocodazole treatment (unpublished data, Fig. 8, 9 and 11; II: Fig. 5 and 6).

Could sheet stabilization then be mediated by other parts of CLIMP-63 or p180 than the microtubule binding domains? Some newly published data suggests that interaction of the luminal coiled coil domains of CLIMP-63 could act as the primary mechanism creating ER sheets (Shibata *et al.*, 2010). However, this is not completely supported by the earlier published data, where increased formation of tubules was a consequence of CLIMP-63 overexpression (Klopfenstein *et al*., 1998). In this respect, p180 is a stronger candidate because its overexpression results in proliferation of stacked ER sheets, which are smooth if the ribosome binding domain is deleted (Wanker *et al*., 1995; Becker *et al*.,1999; Benyamini *et al*., 2009). In addition, p180 depleted cells show lines of short ER profiles (Benyamini *et al*., 2009), which are almost identical to the profiles of fenestrated sheets as shown by us (II). However, smooth and stacked ER cisternae are observed after overexpression of many ER proteins (Wright *et al*., 1988; Amarilio *et al*., 2005; Korkhov and Zuber, 2009). Moreover, the strongest evidence against a function as a master regulator of sheet formation is the fact that both CLIMP-63 and p180 are only expressed in vertebrates (Shibata *et al*., 2009). Thus, these proteins cannot be evolutionarily conserved shaping proteins, but other systems have to exist.

My view is that microtubules possibly play a role in segregating RER and SER as suggested by Nikonov *et al*., (2002; 2007) and assist in stabilizing ER sheets and extending tubules. During interphase, the microtubule “fences” may concentrate polysomes to sheets. When microtubules are removed, enhanced diffusion of the sheet promoting polysomes may spread the sheet areas even to ex-tubular regions. In addition, long ER tubules diminish, because they are not extended or pulled along microtubules anymore. The remaining tubular parts are compact and may contain tightly packed oligomers of membrane bending proteins, which exclude polysomes. Membrane bending proteins are needed for formation of sheet edges as well.

The spreading of the sheet areas depends on the amount of ER-bound ribosomes. If there are plenty, the ribosomal density is not diminished too much even when ribosomes spread over a larger ER area and therefore, the prevailing ER morphology is sheet. During mitosis, when the number of ER-bound ribosomes decreases to variable extent due to inhibition of the initiation stage of translation, ribosomal density on ER is not high enough to stabilize sheets in intact form, but fenestration and tubulation follows.

As such, this is naturally a simplified model that disregards many factors contributing to it. For example, what is the role of actin in these situations and whether it can take over the role of microtubules during mitosis, remains to be seen. In addition, one might argue, that overexpression of reticulon or DP1/REEP proteins is sufficient to convert the ER into a tubular form (Voelz *et al*., 2006; Park *et al*., 2010). It therefore might be that ribosomes have to fit somehow to these tubules. However, the microtubule cytoskeleton shows alterations in response to overexpression of for example REEP 1 protein that is in complex with other proteins shaping the tubular ER network (Park *et al*., 2010). Furthermore, structural identification of the tubular ER in these cases is based on observations with LM, and thus leaves open the possibility that the tubules are in fact larger in diameter than the usual tubules. In support, we have found tubules in Huh-7 cells with a large diameter and a high density of ribosomes (II).

Future perspectives

One of the burning questions I have had throughout my thesis studies is that, how are ER and Golgi structures organized and regulated in more physiological conditions, such as in tissues. Our model systems are cells cultured in 2D and immortalized through viral methods or because they are cancer cells. They are convenient to study but, unfortunately, an incomplete presentation of cells *in vivo*. It is evident that already the new 3D culturing techniques change the patterns of gene expression, cellular functions and cytoskeletal organization profoundly (Horiuchi *et al*., 2009). However, in tissues the cells receive signals not only from the neighbouring cells - like in 3D culture - but from the plasma, and this can further change their functions and structural organization.

Another area of interest is what possibilities the new high resolution LM techniques may open in the future. For example, stimulated emission depletion microscopy has already been proven to resolve tight tubular ER networks that appear as blurred large areas reminiscent of sheets in confocal optical sections of living cells (Hein *et al*., 2008). Such techniques could help to solve discrepancies evoked by the putative inferior preservation of structures with chemical fixation as opposed to *in vivo* imaging or snap freezing techniques (Ladinsky *et al*., 1999; Lu *et al*., 2009). Nevertheless, even in the superresolution LM techniques, visualization of the structures requires the use of fluorophores and thus, EM is still needed to reveal the surrounding cell structures. A well known down side of EM, small sample sizes and volumes, may be solved in the future with the help of new serial block face SEM techniques and automatic segmentation tools allowing visualization of whole cells and even organisms. After organelle structures are characterized in detail with these techniques, we can better associate them with different functions. An understanding of structure-function relationships within organelles may help to detect and ultimately even cure many diseases caused by dysfunctioning organelles.

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